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Title:
Blood-based biomarkers in the maternal circulation associated with fetal growth restriction

Date:
2019-10-01

Citation:
Tong, S., Joy Kaitu'u-Lino, T., Walker, S. P. & MacDonald, T. M. (2019). Blood-based biomarkers in the maternal circulation associated with fetal growth restriction. Prenatal Diagnosis, 39 (11), pp.947-957. https://doi.org/10.1002/pd.5525.

Persistent Link:
https://hdl.handle.net/11343/286174

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Blood-based biomarkers in the maternal circulation associated with fetal growth restriction

Short running title

Biomarkers of fetal growth restriction

Word count: 4,553

Figure count: 1

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pd.5525

Conflict of interest statement

ST, SW and TKL have a commercial agreement with an industry partner to screen for biomarkers for pregnancy complications including fetal growth restriction. ST, SW, TKL and TM hold a provisional patent (#2018901813) relating to circulating biomarkers of placental insufficiency in pregnancy. There are no other conflicts of interest to declare.

Funding statement

National Health and Medical Research Council of Australia (#1160711 #1136418 #1065854). The funding body had no involvement in the writing of this manuscript.

Bulleted statements

- Fetal growth restriction due to uteroplacental dysfunction is a major risk factor for stillbirth, however most affected fetuses remain undetected in the antenatal period
- There are a number of molecules circulating in the maternal blood that correlate with fetal size. Measuring these molecules has potential to better detect at-risk growth restricted fetuses, but a test needs to be identified with stronger diagnostic performance for clinical utility

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ABSTRACT

Fetal growth restriction (FGR) is associated with 3-4 fold increased risk of stillbirth. Identifying FGR, through its commonly-used surrogate - the small-for-gestational-age (SGA, estimated fetal weight and/or abdominal circumference <10th centile) fetus - and instituting fetal surveillance and timely delivery decreases stillbirth risk. Methods available to clinicians for antenatal identification of SGA fetuses have surprisingly poor sensitivity. About 80% of cases remain undetected. Measuring the symphysis-fundal height detects only 20% of SGA fetuses, and even universal third trimester ultrasound detects, at best, 57% of those born SGA. There is an urgent need to find better ways to identify this at-risk cohort.

This review summarises efforts to identify molecular biomarkers (proteins, metabolites or ribonucleic acids) that could be used to better predict FGR. Most studies examining potential biomarkers to date have utilised case-control study designs without proceeding to validation in independent cohorts. To develop a robust test for FGR, large prospective studies are required with *a priori* validation plans and cohorts. Given that current clinical care detects 20% of SGA fetuses, even a screening test with ≥60% sensitivity at 90% specificity could be clinically useful, if developed. This may be an achievable aspiration. If discovered, such a test may decrease stillbirth.

INTRODUCTION

Detecting fetal growth restriction (FGR) is a cornerstone of antenatal care in order to decrease stillbirth risk. Undetected FGR caused by uteroplacental insufficiency is the biggest risk factor for stillbirth in high-income countries[1]. A prospective study of >92,000 pregnancies in the United Kingdom (UK) demonstrated a stillbirth rate of 9.7/1000 for diagnosed FGR defined as customised birthweight <10th centile. If FGR remained unrecognised, the risk more than doubled (19.8/1000)[2].

While uncommon, very preterm (<31 weeks) FGR poses high risk of perinatal death or disability[3]. Once diagnosed, ultrasound and cardiotocograph surveillance and timed delivery reduces morbidity[4] and mortality[5]. Yet half of all stillbirths occurring at viable gestations happen after 34 weeks, including at term[2]. Tragically, at these gestations, infants would likely lead disability-free lives had they simply been safely delivered prior to demise. The absolute risk of stillbirth increases at term[6], with FGR further elevating the risk 3-4 fold[7]. Given trials have consistently shown inducing labour at term does not increase caesarean section rates[8-11], detecting FGR in late pregnancy and offering term induction is a safe and effective intervention to decrease stillbirth risk[12, 13].

Accordingly, improved identification of FGR has been listed as a top 10 priority to reduce stillbirth[14]. It is unfortunate, and perhaps unappreciated, that the overwhelming majority of growth restricted fetuses are missed during pregnancy[2, 15, 16]. In the large UK study, 82% of the growth restricted stillbirth cases were not detected antenatally[2].

The main clinical strategy to screen for FGR is symphysis-fundal height measurement, plus ultrasound for those measuring small, or for those with risk factors for FGR[12, 17, 18]. It is recommended that all women should be assessed at booking for risk factors for a small-for-gestational-age (SGA; estimated fetal weight (EFW), abdominal circumference (AC) or birthweight <10th centile) fetus or neonate in order to identify those who require increased surveillance[12]. Those women with a major risk factor, such as age >40 years, smoking >10 cigarettes a day, cases where either of the parents were SGA infants, cocaine use, daily vigorous exercise, a previous SGA infant or stillbirth, hypertension, diabetes with vascular disease, antiphospholipid syndrome, renal impairment, or heavy bleeding in pregnancy similar to a menses, should have serial ultrasound measurements of fetal size and assessment of wellbeing from 26-28 weeks' gestation[12]. The detection rate for SGA fetuses however, using this time-honoured approach is only 20-30%[2, 19, 20, 15]. With increasing obesity, sensitivity is likely to fall further still[19].

It might be envisaged that third trimester ultrasound estimation of fetal size for all pregnancies might be the answer to detecting the SGA. Surprisingly, the sensitivity of universal ultrasound even in optimal research settings is only 52-57% (90% specificity)[15, 21]. Recent systematic review and meta-analysis of ultrasound at 32 weeks or later found that EFW or AC <10th centile performed with 35-38% sensitivity and for birthweight <10th centile, but with high specificity of 95-97%[22]. The sensitivity of universal ultrasound may be even lower in clinical practice though compared to research settings. Universal third trimester ultrasound, routinely offered in France, identified only 22% of cases of SGA[16]. Despite a low false positive rate of only 2%, perinatal outcomes in France were not seen to be improved for SGA fetuses identified by universal ultrasound due to an increase in iatrogenic preterm

delivery among screen positive pregnancies[16]. Given modest sensitivity, sheer costs, and potential for harm with universal ultrasound[16], it is uncertain whether this should be considered standard of care. To be introduced, appropriately powered implementation studies are required. There is therefore a clinical imperative to find better ways to identify FGR.

This review examines studies that investigate the potential of molecular markers to clinically predict FGR, and its most commonly used proxy, SGA fetuses. Given gestational tissues are generally not accessible, a diagnostic test would most likely sample molecules from maternal blood. The hope is to identify molecules (proteins[23, 24], metabolites, or ribonucleic acids (RNAs)[25, 26]) circulating in altered concentrations in the case of a growth restricted fetus (Figure 1). The placenta or fetus is likely to be the source of potential biomarkers, but they might possibly originate elsewhere, including the maternal endothelium or immune system.

Defining fetal growth restriction

SGA has long been used in most studies as a surrogate for FGR. However, some fetuses are constitutionally small, <10th centile because they are predestined to be. Their placentas function perfectly, they meet their growth potential, and they are not at increased risk of stillbirth. In using SGA as a proxy for FGR, these are erroneously classed as 'cases'. Reciprocally, the SGA definition applies a dichotomous cut-off to what is a continuum of perinatal risk. Large epidemiological studies consistently show stepwise increasing stillbirth risk with decreasing birthweight centiles below the 75th - 97th centile range, where stillbirth rates are lowest[7, 27, 28]. There is accumulating evidence and support for the idea that ≥10th

centile fetuses who slow in growth across pregnancy do so because of placental insufficiency[29-31]. They too, may represent a cohort of FGR at increased stillbirth risk.

Attempts to agree upon definitions to improve detection of true FGR have been made, but no standard definition has consistently been used universally. Recently, consensus definitions have been reached by Delphi procedure for both early-onset and late-onset FGR[31]. Summarised in Table 1, these seem to be the closest to an agreed upon universal definition that the field has come to, to date. However, being SGA on account of an ultrasound EFW, AC, or birthweight of <10th centile remains the most commonly used proxy. EFW <10th centile was the definition used for inclusion in the large "Prospective Observational Trial to Optimise Pediatric Health in IUGR (PORTO) study[32]. The "trial of umbilical and fetal flow in Europe" (TRUFFLE) study defined early-onset FGR as AC <10th centile with elevated umbilical artery resistance, but didn't tackle late-onset disease[33].

While these definitions of FGR[29, 31] are useful, they have been published relatively recently, after many of the biomarker studies discussed throughout this review, and they rely heavily on ultrasound technologies. Therefore, defining cases as SGA for diagnostic research studies is reasonable to screen for new biomarkers, particularly at term. Using SGA as a surrogate for FGR has important pragmatic advantages: it is readily defined without Doppler ultrasound assessment, and with an expected incidence of 10%, the number of cases in cohort studies is relatively high. Most importantly, despite its limitations, SGA does define a high-risk cohort at significantly increased risk of stillbirth[2, 6, 34] and neonatal morbidity[34]. Better detection of the SGA is therefore a worthy pursuit, although it should be kept in mind that it is not synonymous with true FGR. As such, biomarkers to predict SGA should also

correlate with other measures of placental dysfunction and FGR. These might include abnormal Doppler ultrasound parameters[31], low fetal growth velocity[31, 29], and stronger correlations with those at the lowest birthweight centiles.

PROTEIN BIOMARKERS

Proteins of aneuploidy screening tests

Investigators have exploited large clinical datasets generated from aneuploidy screening tests to identify associations between the analytes measured, and SGA infants[35, 23, 36-40]. If particular levels of these analytes could reliably flag pregnancies at higher risk of FGR, these women could be offered ultrasound assessment of fetal growth in later pregnancy. Studies of circulating proteins associated with aneuploidy have been, by far, the largest to examine associations between circulating biomarkers and SGA infants.

For over 15 years, two tests have been offered to screen for fetal trisomy 21 and other aneuploidies[41]. First trimester screening measures serum levels of pregnancy-associated plasma protein A (PAPP-A) and free beta human chorionic gonadotrophin (hCG) at 9-13 weeks' gestation. These are combined with ultrasound nuchal translucency measurement to estimate aneuploidy risk[42]. The second trimester screening test measures free beta hCG, alpha fetoprotein (AFP), unconjugated oestriol and inhibin A concentrations[43, 44]. The associations of these analytes with SGA have each been examined and are summarised in Table 2. PAPP-A has yielded the most consistent correlations.

Of course, the predictive ability of a biomarker changes according to the gestational age at which it is measured. It makes sense that the biomarkers measured in the first and early second trimesters such as those measured in aneuploidy screening may predict cases of FGR that occur due to abnormal placental implantations. However, it would be expected that

these would perform badly to predict cases of FGR occurring due to a normally implanted placenta that develops intrinsic pathologies later in pregnancy.

PAPP-A

PAPP-A, a placenta-derived protein, binds insulin-like growth factors[45] and is thought to be involved in placental function and fetal growth[46]. Many studies have consistently shown significant associations between infant birthweight and first trimester PAPP-A multiples of the median (MoM)[40, 47, 39, 48-54, 35, 55, 56]. PAPP-As <0.4 MoM (<5th centile) incur an odds ratio (OR) of 2.5-2.9 for SGA infants.

While these ORs suggest this may be a worthwhile clinical test, PAPP-A's capacity to predict SGA fetuses is limited. In large cohort studies (n=1,474 - 46,262) PAPP-A <5th centile only predicts 10-36% of cases[39, 40, 48, 49, 56]. The specificity is also poor meaning the vast majority of those with low PAPP-A, will not have a SGA fetus. Overall, the test misses most cases of SGA, with high false positive rates.

Despite this modest predictive value, the Royal College of Obstetricians and Gynaecologists recommends that first trimester PAPP-A <5th centile should prompt serial ultrasound estimation of fetal size because of the increased risk of a SGA fetus[12]. In contrast, the American College of Obstetricians and Gynecologists advises there is no evidence that screening for FGR with PAPP-A improves outcomes[17].

A more extreme PAPP-A MoM cut-off, <1st centile, achieves a highly specific (99%) test with the highest positive likelihood ratios (3.50-3.59) but, unsurprisingly, has an unacceptably low sensitivity of just 3%[35, 39, 56]. While this cut-off is not clinically useful, this data highlights the continuum at play: the lower the PAPP-A MoM, the more likely uteroplacental dysfunction.

First trimester free beta hCG

Low first trimester free beta hCG levels (and high second trimester levels – see supplementary discussion) have also been associated with SGA infants[55, 57-59] but with far less strength and consistency than PAPP-A. In a retrospective study of 34,271 women, first trimester hCG ≤5th centile was associated with SGA infants with an OR of 1.55, but not with birthweight <5th centile[56]. A meta-analysis of first trimester hCG <5th centile in over 11,000 women demonstrated a decidedly modest 6% sensitivity and 96% specificity for SGA[39]; while a more recent meta-analysis suggested low free beta hCG to perform with 34% sensitivity at 90% specificity[35]. Unlike PAPP-A, studies have been conflicting with some large studies failing to find a relationship between low beta hCG and SGA infants at all[48, 54, 40], including one of 49,000 women[40]. It is thus debatable whether clinicians should use low first trimester beta hCG to flag pregnancies as being at increased risk of FGR.

Second trimester aneuploidy screening analytes

While some studies have reported circulating second trimester levels of free beta hCG, AFP, unconjugated Oestriol and Inhibin A to be associated with SGA, none have diagnostic performance characteristics consistent with clinical utility. These are included in the supplementary discussion.

Angiogenesis-related factors

Preeclampsia and FGR are associated with abnormal placental release of angiogenesis-related factors into the maternal circulation. There is reduced pro-angiogenic placental growth factor (PlGF), and increased anti-angiogenic soluble fms-like tyrosine kinase 1 (sFlt-1)[60-64] release. Therefore, PlGF, sFlt-1, the sFlt-1:PlGF ratio, plus other angiogenesis-related

factors, have been investigated as potential biomarkers for FGR. In general, angiogenesis-related factors consistently show a stronger association with preeclampsia than FGR.

PlGF and the sFlt-1:PlGF ratio

PlGF, one of the most studied circulating analytes as a biomarker for FGR, has perhaps the strongest association of any biomarker yet discovered. There is a consistent association between placental dysfunction and low PlGF throughout pregnancy.

Secreted by the placenta, PlGF is classified as pro-angiogenic on the basis of its similarity to Vascular Endothelial Growth Factor (VEGF), which promotes vascular health via receptor mediated signalling on the endothelial cells lining blood vessels[65]. Once in the maternal circulation, PlGF may also signal through the VEGF receptor.

Circulating first trimester PlGF is significantly lower in women destined to deliver a SGA infant[50, 66, 55]. As a lone marker however, it performs poorly with just 27% sensitivity at 90% specificity[35]. Moreover, the addition of first trimester PlGF does not seem to improve the predictive performance of models for SGA that incorporate ultrasound placental volume measurement and uterine artery Doppler findings[66].

PlGF is also significantly lower when measured in the second or third trimester among women who subsequently deliver SGA infants[67, 68, 62, 69-75]. In a large prospective study (n=3,348), low PlGF (<280pg/ml) at 22-26 weeks was associated with an OR of 1.6 for SGA infants without preeclampsia. This increased to 2.7 where abnormal mid-trimester uterine artery Doppler was also present[70]. A pooled meta-analysis concluded PlGF has 46%

sensitivity and 68% specificity for predicting SGA, but included PlGF values sampled at a wide variety of gestations, from first trimester until 29 weeks[76]. This data is difficult to apply since PlGF's accuracy varies according to gestation when measured. In gestation-specific analyses, PlGF performs with 31% sensitivity at 12-18 weeks, and with 23% sensitivity at 24-26 weeks, for 90% specificity – outperforming Inhibin A and the sFlt1:PlGF ratio[68]. PlGF measured at 24 weeks demonstrates 84% sensitivity for 90% specificity for early-onset (<32 weeks) FGR and/or preeclampsia, but predicts late-onset disease poorly (5.3% sensitivity for 80% specificity)[69].

In regards to third trimester PlGF, prospective cohort studies of over 8,200 women showed that 30-34 week PlGF levels were lower among women delivering SGA infants[73], even if they delivered more than 5 weeks after PlGF was measured[72]. Maternal risk factors plus 30-34 week PlGF performed with 58% sensitivity for SGA infants delivered within 5 weeks, and 34% sensitivity for those delivered over 5 weeks later, at 90% specificity (n=9,850)[72]. Unsurprisingly, PlGF levels measured at 35-37 weeks were also significantly lower among women who deliver <5th centile birthweight infants (n=3,895), but PlGF adds minimal predictive power to that of maternal risk factor assessment alone in predicting SGA[74].

Importantly, there is evidence that PlGF may be strongly associated with true placental dysfunction. In a study of SGA fetuses identified by ultrasound, first trimester PlGF <5th centile identified severe placental pathology on histological assessment with 98% sensitivity at 75% specificity[77]. In another, the addition of low first trimester PlGF to high uterine artery pulsatility index (PI) improved sensitivity for FGR, defined as estimated fetal weight (EFW) <10th centile with abnormal umbilical artery (UA) Doppler[50]. In a third study, third trimester

PlGF MoM values were significantly lower among pregnancies with known SGA fetuses who required operative delivery for non-reassuring fetal status, or who suffered neonatal acidosis[78].

Unlike preeclampsia, where the addition of sFlt-1 to PlGF to create the sFlt-1:PlGF ratio performs better as a predictor than either analyte alone[79, 80], sFlt-1 fails to add predictive value to that of PlGF alone for SGA[69, 72, 75, 76, 81]. It seems that sFlt-1 is a biomarker specific to preeclampsia, rather than to FGR[75]. Despite this, recently, a large study evaluated the combination of the sFlt1:PlGF ratio and ultrasound EFW at 28 or 36 weeks' gestation to screen for FGR[82]. Over 3,700 women were included at both gestations. Screen positive was defined as EFW <10th centile and elevated sFlt1:PlGF ratio >85th centile, (>5.78 at 28 weeks; >38 at 36 weeks). EFW <10th centile plus elevated sFlt1:PlGF ratio at 28 weeks had a very high positive likelihood ratio of 41.1, high specificity (99.1%), and a positive predictive value (PPV) of 21.3%, for preterm delivery of a SGA infant. While the combined test outperformed ultrasound EFW <10th and elevated sFlt1:PlGF ratio alone, the high specificity came at a cost of sensitivity. The combined model predicted 38.5% of cases compared to 46.2% predicted by ultrasound alone, and 73.1% predicted by sFlt1:PlGF ratio alone. At 36 weeks the combination of EFW <10th centile plus elevated sFlt1:PlGF ratio again performed with a higher positive likelihood ratio, PPV and specificity than either parameter alone for SGA infants with complications consistent with 'true' FGR. Again however, sensitivity was low at only 37.9% (compared with 67.2% for EFW <10th centile alone, and 53.4% for sFlt:PlGF >38 alone)[82]. A major potential limitation of this study was that one of the complications used to define FGR for the 36 week analysis, was preeclampsia – strongly associated with elevated sFlt1:PlGF ratios. This may have confounded the strong associations observed. The

inclusion of preeclampsia as a definition means the test could be mainly predicting the presence of preeclampsia, and not specifically placental insufficiency leading to FGR.

Overall, PIGF shows a consistent association with SGA infants and uteroplacental dysfunction. While it may not have clinical utility as a lone marker, it may have potential if combined with other diagnostic biomarkers or modalities. Further well designed cohort studies to discover a clinically useful test that incorporates PIGF are justified.

sFlt-1 and soluble endoglin

Summarised in the supplementary discussion, sFlt-1 and soluble endoglin both have very strong associations with preeclampsia, but weaker associations with FGR and SGA infants.

Other protein biomarkers

Other circulating proteins have been reported to have associations with SGA infants or FGR, including Prokineticin1, A disintegrin and metalloprotease 12, Placental Protein 13, Delta-like homologue 1 and Apolipoporotein C-II and C-III_0. Included in our supplementary discussion, none have strong enough performance characteristics to act as a diagnostic test on their own. However, they may have potential to be incorporated into a multi-marker predictive model for FGR.

Multi-parameter predictive models

A number of studies have assessed multi-parameter models combining biomarkers, maternal risk factors and/or ultrasound measures in a bid to better detect SGA. Such models are commonly better at predicting early-onset FGR, rather than SGA fetuses at term. Sensitivity for term SGA infants remains universally poor, unless delivery occurs within 2 weeks of the test[74].

Fadigas et al examined a cohort of 158 birthweight <5th centile infants at term, without preeclampsia, and 3701 controls. Maternal risk factors combined with ultrasound EFW at 35-37 weeks' gestation had 66% sensitivity for 90% specificity for SGA <10th centile. Adding PlGF or sFlt-1 did not improve sensitivity[74]. Combining maternal risk factors with PlGF without ultrasound predicted only 35% of cases[74].

The earlier the test, the poorer the diagnostic performance for predicting term SGA infants. Screening at 30-34 weeks with maternal factors, ultrasound EFW, uterine artery PI, mean arterial pressure and serum PlGF combined predicts only 57% of term SGA infants without preeclampsia (n=9,472)[83]. Uterine artery PI and sFlt-1:PlGF ratio at 24 weeks predicted just 26% of preeclampsia and/or FGR (birthweight <10th centile plus elevated UA PI) occurring at ≥32 weeks, at 90% specificity[69]. Maternal factors, biometry, uterine artery PI, PlGF and AFP combined at 19-24 weeks predicts just 32-33% of term SGA cases without preeclampsia at 90% specificity[71, 84]. A first-trimester prediction model incorporating maternal risk factors, biophysical, and biochemical markers reported 46% prediction of term birthweight <5th centile infants with 90% specificity[55]. The sensitivity would be expected to reduce if assessed for birthweight <10th centile.

While no one has yet found a clinically useful way to identify SGA fetuses with multi-parameter models, it seems an important approach worth pursuing.

METABOLITES AS BIOMARKERS

Besides proteins, other molecules in the maternal circulation include thousands of metabolites – products of cellular metabolism. Examples include amino acids, fatty acids, and sugars. It is entirely possible that in cases of FGR, increased metabolic demand placed upon the placenta might lead to measureable changes in the circulating metabolite profile. Metabolites are typically measured with mass spectroscopy which can quantify thousands of metabolites within each sample.

Using plasma samples collected at 15 weeks in a case-control study of 40 SGA cases and 40 controls, Horgan et al[85] identified a signature of 19 metabolites that produced an area under the receiver operator characteristic curve (AUC) of 0.90 and a very high OR of 44. However, this hopeful finding has not been validated in an independent cohort. If validated, it would imply the remarkable fact that most cases of SGA are biologically predetermined by 15 weeks' gestation.

Heazell et al [86] performed metabolic profiling on serum samples obtained at 28-41 weeks in women presenting with reduced fetal movements. While cases (n=40) were strictly defined as those with poor outcomes, most were SGA at delivery, and all controls (n=40) had birthweights ≥10th centile. This study identified 98 metabolites that significantly differed between the groups. While the AUC values were modest for each of them, a ratio of metabolites produced an AUC of 0.84. Unfortunately there was no validation cohort.

Another interesting case-control study identified a number of metabolites in urine collected at the end of the first trimester associated with FGR[87]. Many had a dose-dependent relationship, making the link more convincing. Unfortunately, this study did not report the diagnostic performance of combining markers, and nor was this data validated.

There may be untapped potential in mining the metabolome. So far there have been few studies, and none have validated their promising leads.

RNA BIOMARKERS

RNAs, including those released from the placenta, circulate in the maternal blood and can be quantitatively measured. It is possible that among pregnancies affected by placental dysfunction and FGR, or those destined to develop FGR, there could be differential expression of placental genes. This could lead to altered release and concentrations of corresponding RNA transcripts in the maternal circulation. These could potentially be measured as RNA-based biomarkers. We have recently reviewed the potential of measuring RNA in maternal blood as a diagnostic test for pregnancy complications, including samples from which RNA can be measured, and the technologies available to measure RNAs[88].

We [89] [90] [91] [92] [93] and others [94] [95] have shown that where FGR is already diagnosed, there are differential changes in circulating messenger RNA (mRNA) [93, 91, 95, 94] and microRNA[92] transcript levels from numerous genes. However, there appears to be only two reports examining the potential of measuring circulating RNAs to identify unsuspected FGR. We

performed a nested case-control study, examining blood samples obtained at 26-30 weeks' gestation. We measured circulating RNA in 80 controls and 40 samples from women destined to deliver a <5th centile infant at term. Using a microarray of RNA transcripts from genes highly expressed in the placenta relative to other tissues, we found 37 to be differentially expressed, validating seven of these with polymerase chain reaction quantification. A combination of four RNAs yielded a respectable AUC of 0.78. However, these case-control findings have not been validated. In a second small prospective cohort (n=52), we showed that Insulin growth factor 2 and Insulin growth factor binding protein mRNAs, from genes known to regulate cellular growth, may be dysregulated at 28 weeks among women destined to deliver a SGA infant at term[90].

Multiple reports have shown changes in circulating RNAs associated with FGR, and the technology to rapidly mine RNAs exists. There is therefore great potential in further interrogating circulating RNAs as biomarkers of FGR. One important barrier is that RNAs are difficult to measure from plasma samples, and cannot be reliably measured in serum[88]. An option to overcome this is to collect blood samples in commercially available tubes that protect RNA in whole blood from degradation[88]. While these tubes are convenient to use, requiring only 2.5ml of blood, they are relatively expensive, making large cohort studies even more costly.

CONCLUSIONS

Unsuspected and undiagnosed SGA fetuses are common[2, 15, 16]. Being SGA incurs a 3-4 fold increased risk of stillbirth; with double the risk for undetected SGA cases compared to their detected counterparts[2]. The combination of fetal surveillance and timely delivery where FGR is diagnosed is a safe, accessible intervention to reduce preventable stillbirth. However, poor antenatal detection of SGA fetuses limit tangible reductions in stillbirth rates. Encouragingly, there seems to be increasing momentum in the development of biomarkers to identify unsuspected FGR, especially in late pregnancy, where half of stillbirths are clustered.

Of all aneuploidy biomarkers, PAPP-A has the most consistent association with SGA infants, but with modest diagnostic performance characteristics. As Non Invasive Prenatal Testing replaces older approaches to aneuploidy screening[96, 97], these crude approaches to screening for pregnancy complications will be used less. Their diagnostic performances for SGA and other pregnancy complications, are too poor to justify their use to screen for these alone.

Of all the circulating markers reported, PlGF remains our best lead. It may have a direct association with uteroplacental dysfunction and seems to aid in the identification of FGR among the SGA. While it is not currently clinically useful on its own, PlGF may have merit if combined with other predictors, such as ultrasound parameters, maternal risk factors, or other biomarkers, that have not yet been discovered or evaluated as predictive algorithms. Furthermore, measurement of circulating metabolomics and RNAs may have untapped potential. Overall however, the use of low-dose aspirin for preterm preeclampsia prevention

in women with risk factors for preeclampsia and/or FGR is becoming more and more the standard of care. The impact of aspirin on the predictive ability of biomarkers is not yet known and may be a significant additional factor to be considered.

We conclude with a few broad observations. First, it would seem unlikely that a lone biomarker will be found that performs with sufficient diagnostic performance on its own. It would be prudent for researchers to embrace multi-marker modelling from the outset in the design of cohort studies. Given the literature thus far, we suggest researchers consider adding PIGF to diagnostic algorithms for SGA infants or FGR.

Secondly, it has been a common theme that studies aiming to develop a diagnostic test for term FGR have been nested case-control studies with no validation of findings. To develop a robust test for FGR, we need to move towards larger prospective studies. These might involve multi-centre collaborations to provide sufficient power, and they should include *a priori* plans to collect a validation cohort.

Finally, the pathophysiology of FGR, and associated perinatal mortality risk, occur according to a continuum. Therefore, potential biomarkers are also likely to be progressively deranged as continuous variables. In contrast, most studies define FGR or SGA as arbitrary dichotomous outcomes. This means there is likely to be a theoretical ceiling for the diagnostic performance of any screening test for SGA or FGR that will be less than perfect. Ideally, studies would be large enough to screen for adverse perinatal outcomes reflective of FGR, and corroborative evidence of biomarkers correlating with various biological manifestations of placental dysfunction would be demonstrated. Likewise, potential

predictors and outcomes should be assessed as continuous variables to identify those at risk across all birthweight centiles.

Despite this, it remains a disturbing fact that current clinical care only detects 20% of SGA cases[15]. Depending on implementation guidelines, the consequence of false positive results from a test for SGA would likely be induction at term. Especially if this was to occur at 39 weeks for cases with no other features suggesting fetal compromise, this does not appear to be harmful[8, 11]. Therefore a diagnostic test for FGR that is clinically useful might not need to be perfect, as long as it is carefully implemented. A theoretical non-invasive screening test for SGA fetuses near term, with ≥60% sensitivity (greater than that of universal ultrasound[15]) and a 90% specificity (equal to universal ultrasound[15]) would be clinically useful. This seems an achievable aspiration. Should such a diagnostic test be developed, it just might decrease the incidence of late pregnancy stillbirth.

TABLES

	Criteria for early-onset FGR (<32 weeks)	Criteria for late-onset FGR (≥32 weeks)
FGR defined by fetal size alone	AC or EFW <3rd centile	AC or EFW <3rd centile
FGR among SGA fetuses with 3rd-10th centile AC or EFW	AEDF in the UA	AC or EFW crossing centiles*
	UA PI >95th centile	UA PI >95th centile
	UtA PI >95th centile	CPR <5th centile
FGR among AGA fetuses	AEDF in the UA	AC or EFW crossing centiles* *and* UA PI >95th centile
		AC or EFW crossing centiles* *and* CPR <5th centile

Table 1: Consensus definitions for early and late-onset FGR among SGA and AGA fetuses

Adapted from "Consensus definition of fetal growth restriction: a Delphi procedure" Gordijn *et al*. *UOG* Vol 48, 2016[31]. AC=abdominal circumference; AEDF=absent end diastolic flow; AGA=appropriate-for-gestational-age (≥10th centile); CPR=cerebroplacental ratio; EFW=estimated fetal weight; FGR=Fetal growth restriction (failure to reach biological growth potential as a consequence of impaired placental function); PI=pulsatility index; SGA=small-for-gestational-age (<10th centile); UA=umbilical artery; UtA=uterine artery; *definition of crossing centiles=crossing >2 quartiles (50 centiles) using non-customised growth centiles.

Table 2: Diagnostic performance summary of proteins measured in first and second trimester aneuploidy screening for birthweight <10th centile

	Trimester	Cut-off	Sensitivity	Specificity
PAPP-A	First	<0.4MoM (<5th centile)	12%[40]	Not reported[40]
	Second		No significant association[71]	
Free bhCG	First	<5th centile	No significant association[40]	
	Second	>2.0MoM	No significant association[98]	
AFP	Second	>2.0 MoM	6%[39]	98%[39]
Unconjugated oestriol	Second	<0.75MoM	37%[39]	88%[39]
		<0.5MoM	2%[39]	99%[39]
Inhibin A	Second	>2.0MoM	11%[39]	98%[39]

AFP = alpha fetoprotein; bhCG = beta human chorionic gonadotrophin; MoM = Multiples of the median; PAPP-A = Placenta associated plasma protein A.

FIGURE LEGEND

Figure 1: Overview of circulating proteins, metabolites and Ribonucleic Acids (RNAs) and how they are measured

The placenta experiencing inadequate perfusion is likely to undergo adaptive changes, resulting in differential expression of placental genes and increased or decreased generation of their related proteins, products of metabolic processes, and RNA transcripts for which they encode. These potential 'placental dysfunction signature molecules' are released from the placenta into the maternal circulation where they can be measured. This is the basis of a potential biomarker test for fetal growth restriction.

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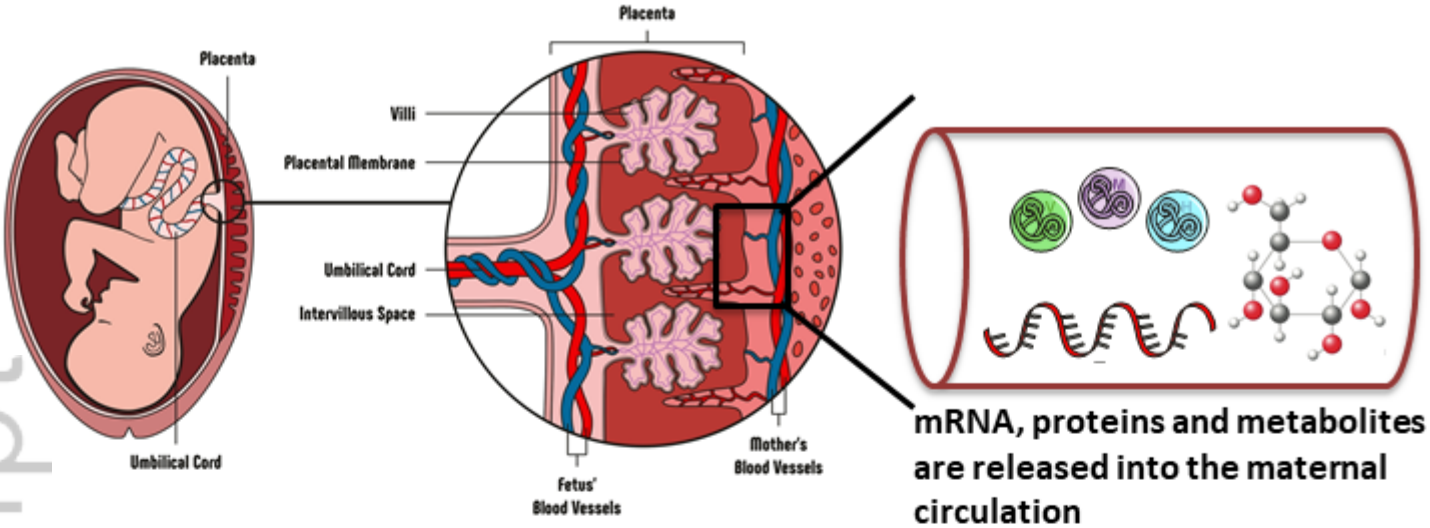
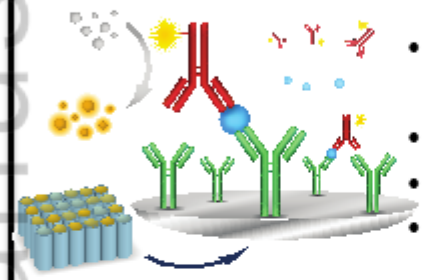
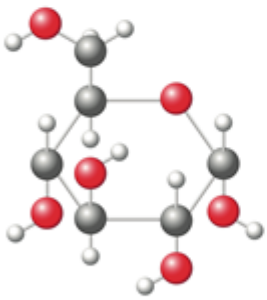


Measuring proteins in the blood



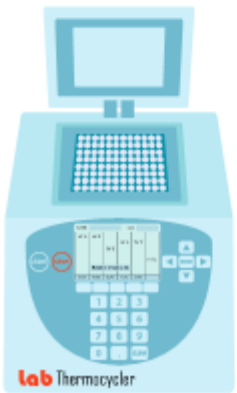
- Proteins are released from the placenta into the maternal blood
- Proteins can be measured in the separated plasma or serum
- Specific proteins can be measured by ELISA
- ELISA uses antibodies to pick out proteins from the plasma or serum and measure their levels

Measuring metabolites in the blood



- Metabolites are products of metabolism that are released into the maternal blood
- Samples are assessed in a mass spectrometer
- A mass spectrometer ionizes samples into ions that are identified based on their mass and charge
- High resolution mass spectrometry systems and enhanced metabolite databases/libraries for comparison allow accurate identification of metabolites in the blood

Measuring RNA in the blood



- Micro-RNA (miRNA) and messenger RNA (mRNA) are released from the placenta into the maternal blood
- miRNA or mRNA are extracted from whole blood
- Specific genes of interest can be measured by polymerase chain reaction (PCR)
- Whole genome (identification of all RNAs) can be assessed using next generation sequencing

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