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EBioMedicine

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Research paper

Maternal plasma miRNAs as potential biomarkers for detecting risk of small-for-gestational-age births

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ARTICLE INFO

ABSTRACT

Background: Small-for-gestational-age fetuses (SGA) (birthweight <10th centile) are at high risk for stillbirth or long-term adverse outcomes. Here, we investigate the ability of circulating maternal plasma miRNAs to determine the risk of SGA births.

Methods: Maternal plasma samples from 29 women of whom 16 subsequently delivered normally grown babies and 13 delivered SGA (birthweight <5th centile) were selected from a total of 511 women recruited to form a discovery cohort in which expression data for a total of 800 miRNAs was determined using the Nanostring nCounter miRNA assay. Validation by RT-qPCR was performed in an independent cohort.

Findings: Partial least-squares discriminant analysis (PLS-DA) of the Nanostring nCounter miRNA assay initially identified seven miRNAs at $12-14^{+6}$ weeks gestation, which discriminated between SGA cases and controls. Four of these were technically validated by RT-qPCR. Differential expression of two miRNA markers; hsa-miR-374a-5p ($p = 0•0176$) and hsa-let-7d-5p ($p = 0•0036$), were validated in an independent population of 95 women (SGA $n = 12$, Control $n = 83$). In the validation cohort, which was enriched for SGA cases, the ROC AUCs were $0•71$ for hsa-miR-374a-5p, and $0•74$ for hsa-let-7d-5p, and $0•77$ for the two combined.

Interpretation: Whilst larger population-wide studies are required to validate their performance, these findings highlight the potential of circulating miRNAs to act as biomarkers for early prediction of SGA births.

Funding: This work was supported by Genesis Research Trust, March of Dimes, and the National Institute for Health Research Biomedical Research Centre (NIHR BRC) based at Imperial Healthcare NHS Trust and Imperial College London.

1. Introduction

Small-for-gestational-age (SGA) refers to infants born with a birth weight of less than the 10th centile defined against population centiles for that gestational age. Approximately 27% of all live births in low- and middle-income countries have been reported to be SGA [1]. However, evidence derived from personalised growth charts suggest that SGA may actually reflect appropriate growth for $50-70\%$ of fetuses at birth when important maternal information such as BMI and ethnicity are accounted for [2]. In contrast, fetal growth restriction (FGR) reflects a restriction of growth potential, often due to placental pathology. Severe SGA babies are more likely to be growth restricted, and have a higher risk of perinatal morbidity and mortality [3,4] in addition to long-term complications such as an increased risk of the development of cardiovascular and cerebrovascular diseases [5], and metabolic disorders such as non-insulin-dependant diabetes mellitus [6].

In current clinical practice, women with existing risk factors of SGA are screened using ultrasound-based approaches to identify SGA babies before birth. Thus, these approaches have limited sensitivity and specificity resulting in many of SGA babies not being detected before birth [7]. Furthermore, discriminating SGA and FGR by ultrasound ideally requires serial growth scans from early in pregnancy. Therefore, there is a clinical need to develop a robust and reliable approach for risk prediction of SGA and FGR in early pregnancy.

MicroRNAs (miRNAs) are small single-stranded, non-coding RNAs of 19 - 22 nucleotide in length which regulate the expression of

Research in Context

Evidence before this study

Stillbirth is one of the main causes of mortality worldwide. Around half of all stillbirths occur in fetuses that are small-for-gestational-age (SGA). Maternal abdominal palpation and measurement of symphysio-fundal height detects less than one third of SGA neonates in routine clinical practice. Although a routine third trimester scan is superior in identifying these pregnancies, a recent systematic review by the National Institute for Health and Care Excellence (NICE) concluded that methods currently used for SGA screening are poorly developed, do not improve outcome and therefore represent research priorities. Screening for SGA is a challenge particularly in low-risk pregnancies hence biochemical markers reflective of placenta insufficiency represent attractive methods to detect women at risk of adverse pregnancy outcomes.

Added value of this study

There is a growing focus on circulating miRNAs as prognostic/diagnostic biomarkers due to their stability and the non-invasiveness of sample collection. In this study, we initially identified in a discovery cohort seven circulating miRNAs differentially expressed in the early second trimester between SGA cases and normal controls. From these, two miRNAs (hsa-miR-374a-5p and hsa-let-7d-5p) were validated to be discriminatory in an independent cohort by RT-qPCR. The combined relative expression of these two miRNAs demonstrated predictive ability for SGA cases with a ROC AUC of 0.77 (95% CI 0.601−0.943), although the validation cohort was enriched for SGA cases compared to that expected in an entirely low-risk population.

Implications of all available evidence

Current screening practices fail to detect the majority of SGA babies prior to birth. Our study identifies circulating miRNAs as potential molecular biomarkers for SGA births, which may facilitate early risk stratification and targeted surveillance and intervention strategies. Larger population-wide studies are required to further validate their performance.

target mRNAs at post-transcriptional and translational level [8]. They have been implicated in the regulation of various processes including inflammation [9], cell proliferation, cell differentiation [10] and apoptosis [11], with a single miRNA able to alter the expression of thousands of genes. As such, there exists much interest in their utility as potential biomarkers for pathophysiology and disease. The diagnostic potential of miRNAs has been investigated in several pregnancy-related complications such as preeclampsia [12], ectopic pregnancy [13], gestational diabetes [14], recurrent pregnancy loss [15] and pre-term delivery [16-18]. To date there have been two main approaches used to identify potential miRNA biomarkers for SGA; the measurement of previously reported candidate miRNAs in the placenta using real-time quantitative RT-PCR (RT-qPCR) [19] or untargeted profiling of maternal circulating miRNAs using microarrays [20]. Both methods have limited sensitivity in comparison to direct digital quantification technology by Nanostring nCounter miRNA profiling assay [21], which utilises hybridization-based methods to directly quantify target sequences without the need of an amplification step thus enabling more robust quantification of less abundant miRNAs. In this study, we used Nanostring nCounter miRNA profiling to identify circulating plasma miRNAs in the early second trimester of pregnancy that are predictive of SGA.

2. Methods

2.1. Recruitments and sample collection

The participants were pregnant women attending antenatal clinics at Imperial College Healthcare NHS Trust Hospitals and Chelsea & Westminster Hospital in London, UK. The exclusion criteria included women with multiple pregnancies, women with pre-existing diseases, and those who subsequently developed other obstetric complications including pre-eclampsia (de-novo hypertension present after 20 weeks' gestation combined with proteinuria PCR> 30 mg/mmol) [22], gestational hypertension (systolic hypertension >140 mmHg and diastolic blood pressure (DBP) >90 mmHg on two occasions, more than 4−6 hrs apart, or one reading >110 mmHg) [23], gestational diabetes, preterm birth, and obstetric cholestasis. Maternal plasma samples were extracted from whole blood and collected prospectively at three time-points; $12^{+0}-14^{+6}$ (time-point A), $15^{+0}-17^{+6}$ (time-point B) and/or $18^{+0}-21^{+6}$ (time-point C) weeks gestation. Whole blood was processed for plasma as described by Cook et al. [18]. Isolated plasma was stored in aliquots at −80 °C until further analysis. Following delivery, the samples were divided into SGA cases, defined as birth-weight below the 5th centile for gestational age using customised growth charts based on parity, baby gender, ethnicity, and average height and weight for each ethnic groups, and controls with birth-weights appropriate for gestational age (10th - 90th centile) [24].

2.2. Ethics statement

Ethical approval for the discovery study was obtained from the Hertfordshire Research Ethics Committee (reference: 11/H0311/6). The biological validation study was approved by the London − Stanmore Research Ethics Committee (reference: 14/LO/0328). Written informed consent was obtained from all participants.

2.3. RNA extraction

Plasma aliquots were thawed on ice prior to centrifugation at 800 x *g* for 10 min at 4 °C, and only the upper 750 μl of plasma was used for the RNA extraction to avoid cellular and/or platelet contamination [25]. RNAs were obtained with the Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit (Slurry Format) (Norgen Biotek, Ontario, Canada) according to the manufacturer's recommendations. In accordance with the protocol, 5000 attomoles of the spike-in control, cel-miR-254 (Exiqon, Vedbaek, Denmark), was added to plasma following lysis and denaturation to allow normalization of any technical variation that may occur during the RNA extraction process. The RNA was further concentrated and purified using the Amicon Ultra YM-3 columns (Merck Millipore, Darmstadt, Germany) as recommended.

2.4. nCounter miRNA assay and data analysis

Extracted RNAs were subjected to nCounter™ plasma miRNA profiling (Nanostring, Seattle, USA) which allows direct assessment of ~800 human miRNA target expression levels without amplification steps [26]. The pre-built target miRNAs of nCounter™ miRNA profiling assay include 98% of miRNAs from miRBase v22 (http://mirbase.org/) that have been sequenced with high confidence. The resulting counts were analysed using the nSolver (v3.0, Nanostring, Seattle, USA). Raw counts from nCounter were normalized to the expression of the top 100 highly expressed miRNAs. Only those miRNAs expressed above background level in greater than 50% of samples from any outcome group were used for further analysis. Background level was defined by 2-standard deviations (SD) above the mean negative control counts. The top 50 most abundantly expressed miRNAs

were used for further analysis to eliminate the 'run-dependent' variation from those miRNAs which had extremely low expression in the plasma samples. All raw count data are available in Supplementary data.

Unsupervised hierarchical clustering of miRNA profiles was performed using Ward clustering [27] in ClustVis (https://biit.cs.ut.ee/clustvis/) [28]. Additional multivariate analyses were carried out using SIMCA-P (soft independent modelling of class analogies-P, v 14.0, Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used to examine data structure and covariance in the miRNA profiles of samples. Supervised partial least-squares discriminant analysis (PLS-DA) was performed to determine the capacity of circulating miRNAs profiles to discriminate control and SGA cases. PLS-DA model robustness and fit were assessed by examining R^2 (correlation) and Q^2 (predictability) coefficients as well as response to permutation testing ($n = 100$) whereby R^2 and Q^2 coefficient degradation to <0.3 and <0, respectively, was used to confirm robust modelling without data overfitting [29]. Plasma miRNAs most responsible for discrimination of SGA cases from controls were identified by variable importance for projection (VIP) scores of >1 in the PLS-DA model.

2.5. Real-time quantitative polymerase chain reaction (RT-qPCR) and data analysis

For technical validation of the discovery study, and the independent biological validation study, RT-qPCR was used to determine the relative expression of each miRNA. Reverse transcription of extracted plasma RNA was performed using miRCURY LNA™ Universal RT miRNA cDNA synthesis kit II (Exiqon, Vedbaek, Denmark) with the addition of UniSp6 (10^8 copies/μl) to allow normalization of variation that may occur during the reverse transcription process. A total of 2 μl RNA, corresponding to the mass of RNA derived from approximately 60 μl of starting plasma, was used for the reverse transcription reaction following manufacturer's instructions.

RT-qPCR was performed using the custom pick and mix panels with LNA™ primers (Exiqon, Vedbaek, Denmark). The ExiLENT SYBR® Green master mix (Exiqon, Vedbaek, Denmark) was used as per manufacturer's instructions and the RT-qPCR reactions were carried out on an ABI StepOnePlus Real Time PCR System (Life Technologies, Paisley, UK). The cycle conditions for miRNA targets included initial polymerase activation/denaturation step at 95 °C for 10 min, followed by 45 cycles of amplification step consisting of 95 °C for 10 s and 60 °C for 1 min. Melt curve analyses were used to confirm a single PCR product. Raw fluorescence data were collected by the StepOne software v2.3 (Life Technologies, Paisley, UK) and exported for further analyses.

The LinRegPCR program v2017.1 [30] was used to determine Cq values and primer efficiencies for all samples and miRNA targets. Preprocessing of Cq data consisted of normalization to the interplate calibrator, RNA extraction control, cel-miR-254, and the reverse transcription control, UniSp6. To evaluate the miRNA expression, Cq values from LinRegPCR were normalized to two endogenous controls and fold differences to control samples were obtained ($2^{-\Delta Cq}$).

The two endogenous miRNA controls were identified from the nCounter assay data using NormFinder [31] which incorporates the inter- and intra-group variances and calculates the stability value per miRNA target as a measure of expression stability. It also identifies the best combination of miRNA targets to provide the highest stability. ΔCq values obtained from SGA cases and controls were subsequently used for univariate analyses.

2.6. Statistics

The Nanostring nCounter miRNA assay counts were compared between classes using a *Mann-Whitney U test* (GraphPad Prism 5, GraphPad Software, CA, USA) with a *p*-value of <0.05 used to consider a significant difference. Count data are plotted as median and interquartile range.

For Univariate analyses of RT-qPCR results, data were initially tested for normality using *Kolmogorov-Smirnov* test and unpaired *t*-test was used to compare two groups of normally distributed data. For non-parametric, *Mann-Whitney U test* (2-tailed) was used where a *p*-value of <0.05 was considered to be statistically significant. Receiver operating characteristic (ROC) curves were generated using GraphPad Prism 5 (GraphPad Software, CA, USA) for individual miRNAs, and SPSS 26 (IBM, Chicago, USA) for multiple miRNAs. Relative expression ($2^{-\Delta Cq}$) were plotted as median and interquartile range.

2.7. Role of funding source

Funders had no role in the study design, data collection, data analysis, interpretation, or writing of report.

3. Results

3.1. Elucidation of plasma miRNA profiles in SGA cases and controls

Our discovery study cohort consisted of 511 pregnant women recruited during their second trimester. From this cohort, plasma samples were prospectively collected at three time points ($12^{+0}–14^{+6}$, $15^{+0}–17^{+6}$ and $18^{+0}–21^{+6}$ weeks). From 511 women, 13 women subsequently had SGA ($<$5th centile). Total 16 women who had serial plasma sample collection and had normal weight babies were selected for nCounter miRNA profiling assay. The demographic and clinical characteristics of both groups were similar except for birth-weight ($p<0.001$, *Mann-Whitney U test*) (Table 1).

A total of 414/800 (52%) plasma miRNAs were detected with expression levels above background by the nCounter profiling assay. Hierarchical clustering of the top 50 most abundantly expressed miRNAs showed a clear separation of SGA cases from controls that was independent of time-point of sample collection (Fig. 1). Similarly, unsupervised principal component analysis (PCA) showed clear discrimination of SGA cases from controls in the first two principle components of the miRNA data regardless of gestation of sample (Fig. 2a). Supervised partial least-squares discriminant analysis (PLS-DA) was then used to identify miRNAs most strongly associated with SGA (Fig. 2b). The resulting PLS-DA model provided robust separation of SGA cases from controls (R^2(X) = 0.619, R^2(Y) = 0.787, Q^2 = 0.756 for two component model), which were validated using internal cross validation and permutation testing ($p<0.001$) (Fig. 2c). Six discriminatory nCounter probes with Variable Importance in the Projection (VIP) scores >1 were identified and shown to contribute both positive and negative regression coefficients to the PLS-DA model (Fig. 2d

Table 1
Demographic and clinical characteristics of the discovery cohort.

Demographic and Clinical variables of the Discovery cohort			
	Controls (n = 16)	SGA (n = 13)	*p*-value*
Maternal age (SD)	35.9 (5.88)	32.9 (5.91)	0.1804
Ethnicity (%)			0.2071
Caucasian	8 (50)	8 (61.5)	
Asian	4 (25)	2 (15.4)	
Black	4 (25)	3 (23.1)	
Gestational age at delivery (SD)	38.6 (4.24)	37.7 (3.61)	0.1105
Baby gender (%)			0.2029
Male	7 (43.75)	7 (53.85)	
Female	9 (56.25)	6 (46.15)	
Birthweight (SD)	3455 (388.9)	2241 (613.1)	<0.0001
Birthweight Centile (SD)	51.9 (30.7)	2.4 (1.6)	<0.0001

* *p*-value corresponds to Mann-Whitney *U* test (continuous) or Chi-squared (categorical) for the difference in study participants' demographic and clinical characteristics between control and SGA groups.
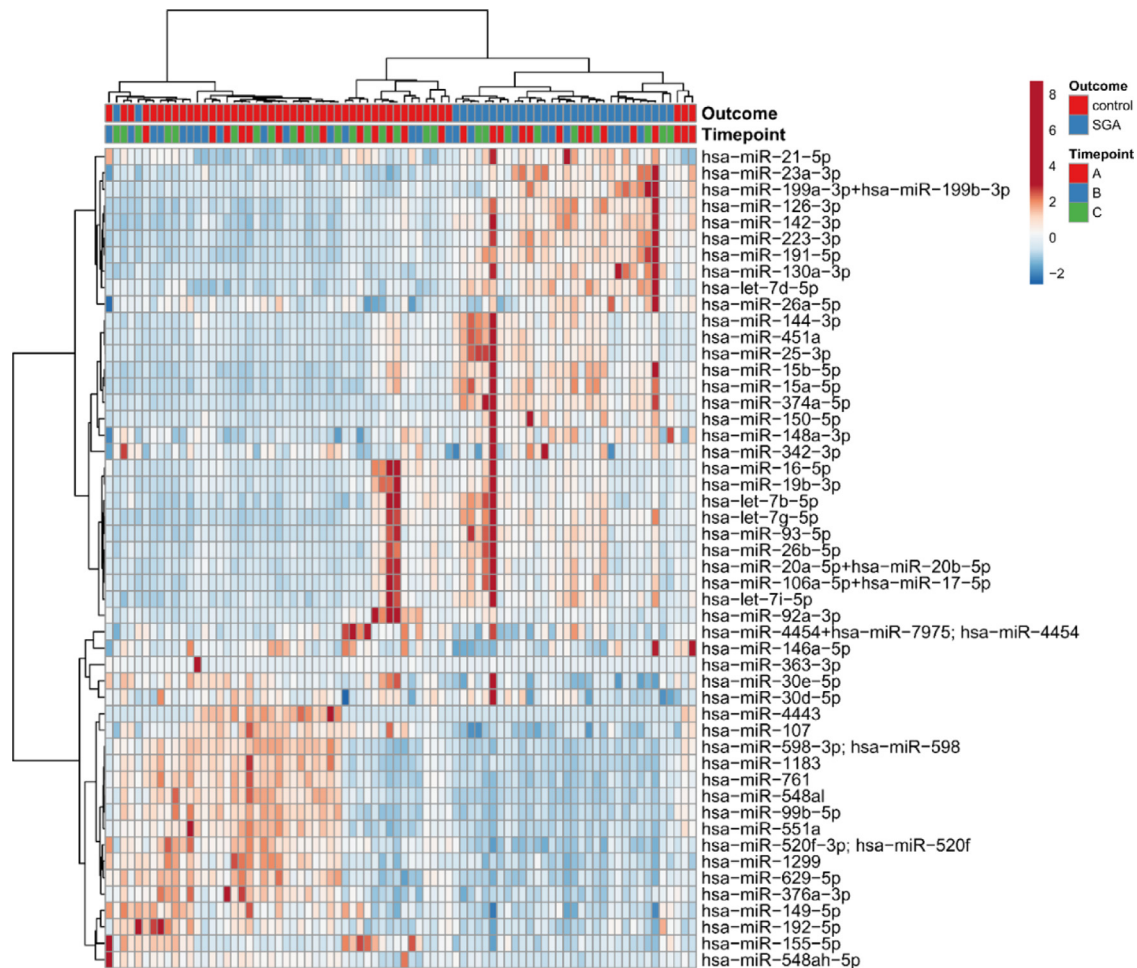
Fig. 1. SGA is associated with a consistently altered plasma miRNA profile across gestation. Hierarchical clustering heat map showing expression profiles for top 50 most abundant plasma miRNAs from SGA ($n = 13$) and controls ($n = 16$) detected by Nanostring nCounter miRNA assay. Clear discrimination of SGA and control patients could be observed regardless of the sampling gestation time point.

and e). Multivariate modelling at each sampling time-point confirmed strong discrimination of SGA and controls across gestation (Supplementary Figure S1). Six nCounter probes (one of which cross hybrids with two miRNA targets) were identified to contribute the majority of discrimination between SGA cases and controls in each of the three time-points assessed throughout gestation (Table 2). Levels of hsa-miR-374a-5p, hsa-miR-191−5p, and hsa-let-7d-5p, were higher in SGA cases whereas hsa-miR-107, hsa-miR-30e-5p and hsa-miR-4454+miR-7975 were lower in SGA cases compared to controls (Fig. 2e).

At time-point A ($12^{+0}−14^{+6}$ weeks of gestation), hsa-miR-374a-5p, hsa-miR-191−5p, and hsa-let-7d-5p, were significantly increased in SGA cases ($p<0.001$ vs controls, Mann-Whitney U test; Fig. 3a-c) whereas hsa-miR-107, hsa-miR-30e-5p and hsa-miR-4454+miR-7975 were decreased compared to controls ($p<0.001$, $p = 0.0048$, $p = 0.047$ vs controls, Mann-Whitney U test; Fig. 3d-f). Differential expression of these miRNA markers was also observed at time-points B ($15^{+0}−17^{+6}$ weeks) and C ($18^{+0}−21^{+6}$ weeks) (Supplementary Figure S2).

3.2. Validation of nCounter miRNA profiling assay data from time-point A using real-time qPCR

We performed technical validation of candidate plasma miRNAs using RT-qPCR. Data were normalized to both synthetic spike-ins, cel-miR-254 and Unisp6, as to account for potential technical variation due to differences in RNA extraction and reverse transcription, and endogenously expressed miRNAs identified in the nCounter

assay using the NormFinder algorithm [31]. For endogenous controls, the two miRNAs with the least variation across the dataset were identified which were hsa-miR-30d-5p and hsa-let-7i-5p. From the seven miRNA targets identified using the nCounter assay, four were technically validated; hsa-miR-374a-5p ($p = 0.0003$, Unpaired t-test), hsa-let-7d-5p ($p = 0.0031$, Unpaired t-test), hsa-miR-4454 ($p = 0.0009$, Mann-Whitney U test) and hsa-miR-7975 ($p = 0.0012$, Mann-Whitney U test) (Fig. 4a-d). RT-qPCR failed to validate hsa-miR-191, hsa-miR-107 and hsa-miR-30e-5p (Fig. 4e-g).

Following a technical validation, we performed a biological validation by RT-qPCR in an independent patient cohort using maternal plasma at 12^{+0} - 14^{+6} weeks gestation. A total of 674 women had been recruited, of whom 341 women had plasma samples collected between 12^{+0} and 14^{+6} weeks gestation and therefore used for validation. Following the exclusion of women who withdrew/were lost during the study follow-up and those who subsequently experienced pregnancy-associated complications such as gestational hypertension, pre-eclampsia and preterm birth, 12 women went on to deliver SGA babies (<5th centile). We then selected 83 women who delivered normal weight babies at term to form our control group that is matched for maternal age, ethnicity, gestational age at delivery, and baby gender ratio.

Of the 95 women included in the validation study, 12 had SGA and 83 had normal weight babies. Similar to the discovery cohort, the demographic and clinical characteristic of both SGA and normal control groups were comparable except for the birthweight ($p<0.0001$, Mann-Whitney U test), as expected (Table 3). Significantly higher
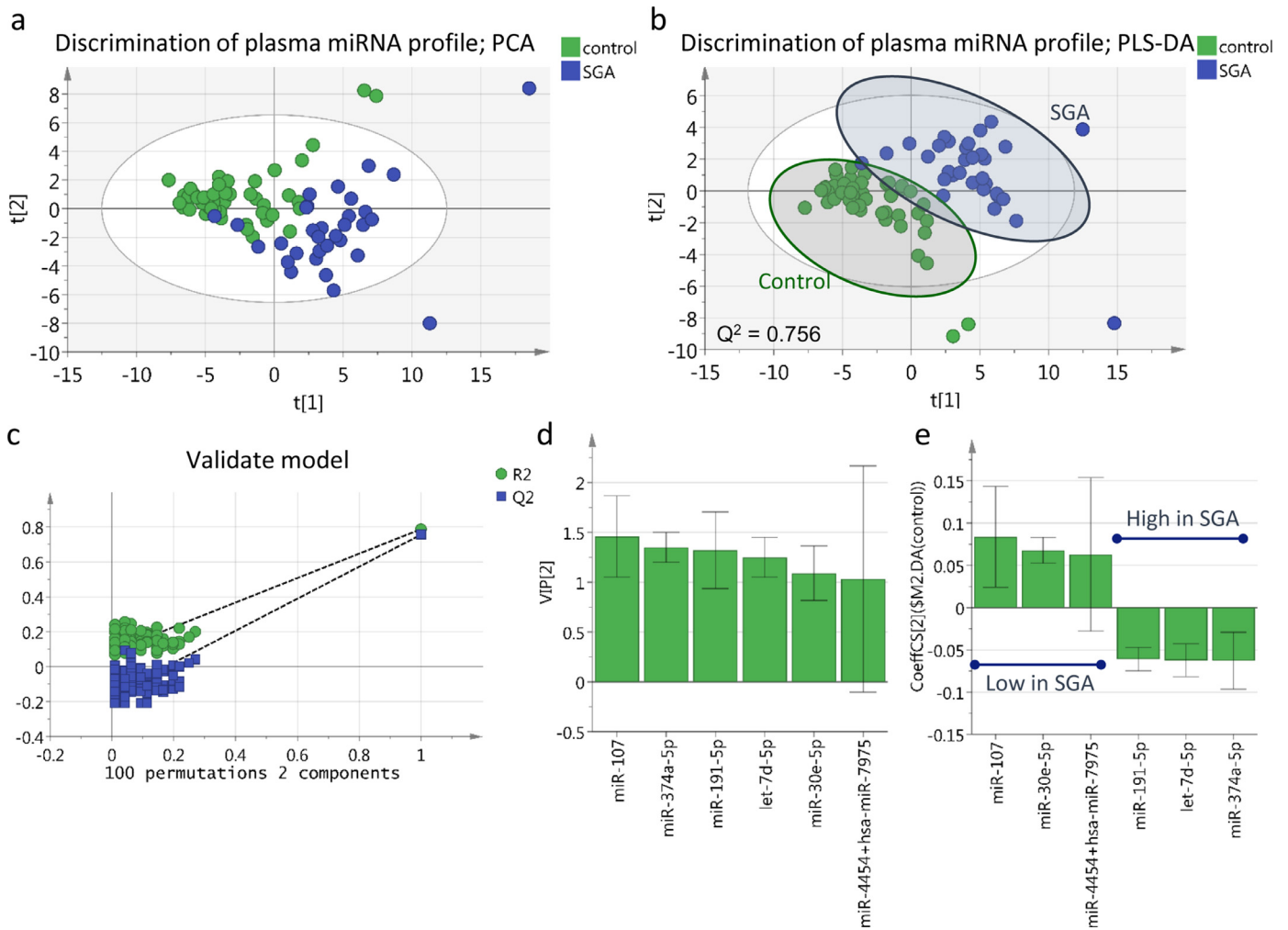
Fig. 2. Multivariate modelling of plasma miRNA expression of women who delivered SGA babies and those with normal weight babies. **(a)** PCA score plot of top 50 most abundant plasma miRNAs. **(b)** PLS-DA score plot shows clear clustering of SGA and control groups. **(c)** PLS-DA model validation by comparing to the classification statistics of models generated after random permutations (100) of the data shows clear R^2 and Q^2 degradation to below 0.3 and 0, respectively. **(d)** Variable importance in the projection (VIP) plot of the PLS-DA model shows six identified miRNA markers which are important in discriminating between miRNA profiles of the two groups. **(e)** Regression coefficient plot of PLS-DA model indicating most important miRNA expression changes (increase/decrease) in SGA compared with control.

expression of hsa-miR-374a-5p (p = 0.0176, *Mann-Whitney U test*) and hsa-let-7d-5p (p = 0.0036, *Unpaired t-test*) were observed in plasma from women who subsequently delivered SGA babies (Fig. 5a and b). Hsa-miR-4454 and hsa-miR-7975, which were technically validated in the discovery cohort, failed to validate in the independent patient cohort (Fig. 5c and d).

The ability of these miRNAs to predict SGA was then assessed using receiver operating characteristic (ROC) analysis using the RT-qPCR data of the validation cohort at 12^{+0} - 14^{+6} weeks gestation. Those miRNAs highly expressed in SGA, hsa-miR-374a-5p and hsa-let-7d-5p, demonstrated good predictive ability for SGA individually with ROC area

under the curve (AUC) of 0.71 and 0.74, respectively (Fig. 6a and b). The strongest predictive ability was achieved when the relative expression of hsa-miR-374a-5p and hsa-let-7d-5p were combined with AUC of 0.772 (95% CI 0.601−0.943) (Fig. 7). This suggests the potential additive predictive value of additional miRNAs or other clinical biomarkers/risk factors, although this requires further investigation.

4. Discussion

Early detection and diagnosis of SGA remains a major challenge in obstetric practice, especially for discriminating SGA babies that

Table 2
Plasma miRNAs differentially expressed in SGA cases compared to controls at different time points. The miRNAs listed are top six miRNAs contributing to the characterization of patient outcome which were identified using the nCounter miRNA profiling assay data. TPs: Time points.

|	Target miRNAs significant in classification (outcome)			
50miRNAs (All TPs)	50miRNAs (TPA: 12−14 weeks)	50miRNAs (TPB: 15−17 weeks)	50miRNAs (TPC: 18−21 weeks)	miRNA counts
miR-374a	miR-374a	miR-374a	miR-374a	High in SGA
Let-7d	miR-23a	Let-7d	Let-7d	
miR-191	miR-191	miR-191	miR-191	
miR-107	miR-107	miR-155	miR-107	High in Controls
miR-30e	miR-30e	miR-16	miR-30e	
miR-4454+miR-7975	miR-4454+miR-7975	miR-4454+miR-7975	miR-548al	
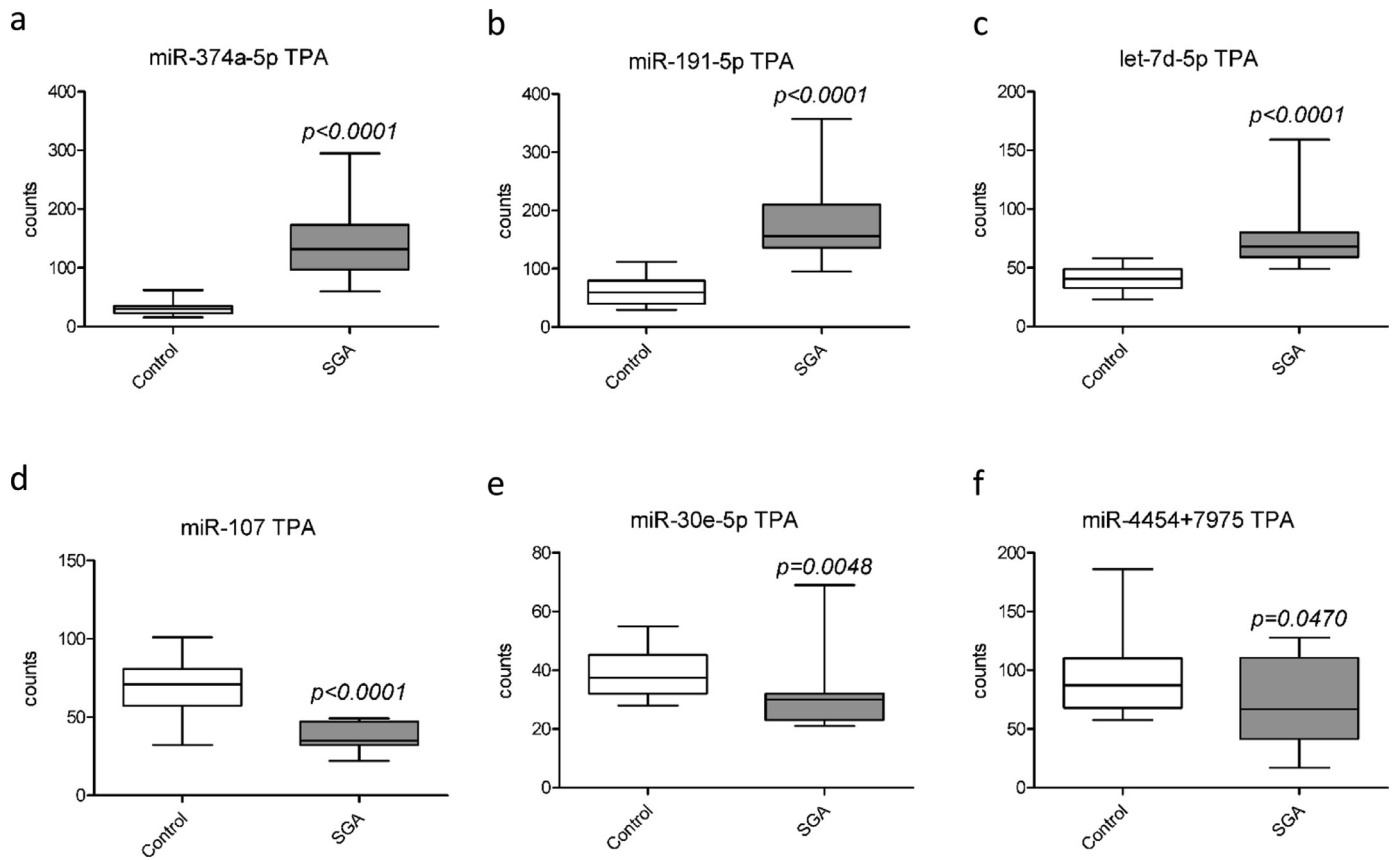
Fig. 3. Top 7 discriminatory miRNAs in early second trimester maternal plasma ($12^{+0}-14^{+6}$ weeks). nCounter probe counts for (a) hsa-miR-374a-5p, (b) hsa-miR-191, (c) hsa-let-7d-5p, (d) hsa-miR-107, **(e)** hsa-miR-30e-5p, and **(f)** hsa-miR-4454+hsa-miR-7975 detected in human maternal plasma in the early second trimester ($12^{+0}-14^{+6}$ weeks, time point A) in women who subsequently delivered SGA babies ($n = 11$) or normal weight babies ($n = 16$). Statistical significance of differences in mean read counts between groups was tested using the *Mann-Whitney U test*. Graphs show median and interquartile range.
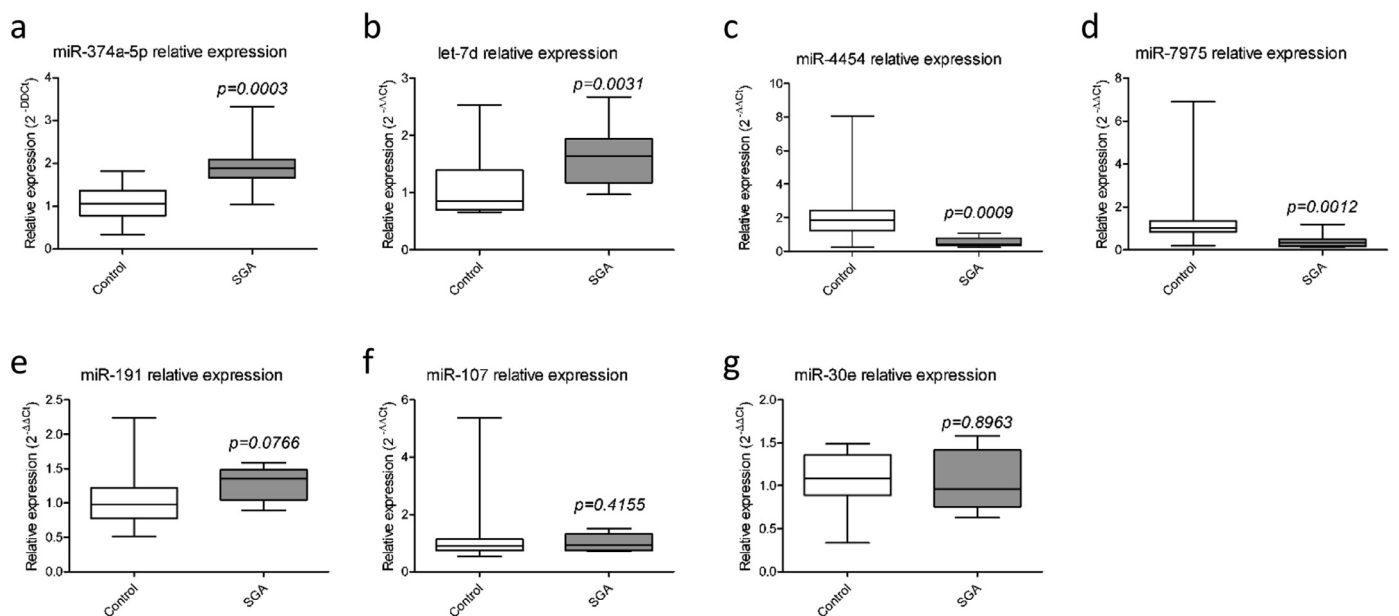

Fig. 4. Technically validated miRNAs differentiating SGA and control samples at time point A ($12^{+0}-14^{+6}$ weeks) in the discovery cohort. Total RNA were extracted from plasma samples and used to technically validate candidate miRNA expression using RT-qPCR. Resulting Cq values normalized for any discrepancies in the efficacy of RNA extraction using the spike-in cel-254 (5000attomoles) and for reverse transcription using the spike-in UniSp6. Two miRNAs, hsa-miR-30d-5p and hsa-let-7i-5p, identified using NormFinder were used as endogenous controls. All values were then normalized relative to the average of the control samples. *P* values were determined by *Mann-Whitney U test*. The differential plasma levels of **(a)** hsa-miR-374a-5p, **(b)** hsa-let-7d-5p, **(c)** hsa-miR-4454 and **(d)** hsa-miR-7975 were validated using RT-qPCR. Hsa-miR-191 **(e)**, hsa-miR-107 **(f)** and hsa-miR-30e-5p **(g)** in SGA patients were not significantly different compared to normal controls (Control $n = 16$, SGA $n = 11$, *Mann-Whitney U test*). Graphs show median and interquartile range.

Table 3
Demographic and clinical characteristics of the validation cohort.

Demographic and Clinical variables of the Validation cohort			
	Controls (n = 83)	SGA (n = 12)	p-value*
Maternal age (SD)	33 (4.35)	35 (3.89)	0.1979
Ethnicity (%)			0.8026
Caucasian	53 (63.9)	8 (66.7)	
Asian	20 (24.1)	2 (16.7)	
Black	10 (12)	2 (16.7)	
Gestational age at delivery (SD)	38.7 (1.79)	39.2 (1.18)	0.4324
Baby gender (%)			0.3485
Male	50 (60.2)	5 (41.7)	
Female	33 (39.8)	7 (58.3)	
Birthweight (SD)	3524 (497.3)	2548 (206.6)	<0.0001

* p-value corresponds to Mann-Whitney U test (continuous) or Chi-squared (categorical) for the difference in study participants' demographic and clinical characteristics between control and SGA groups.

are pathologically small and constitutionally small. In this study, we have focused on SGA threshold of less than 5th centile to identify miRNA markers that can better distinguish pathologically small babies. Here, we report the identification of seven miRNAs whose expressions in early pregnancy is predictive of subsequent SGA birth. Four of the identified miRNAs were technically validated using RT-qPCR; hsa-miR-374a-5p, hsa-let-7d-5p, hsa-miR-4454 and hsa-miR-7975. The expression of these miRNA markers were examined in an independent population where hsa-miR-374a-5p and hsa-let-7d-5p were expressed significantly higher in SGA cases compared to normal controls. The relative expression of hsa-miR-374a-5p or hsa-let-7d-5p alone exhibited good prediction for SGA in the early second trimester (AUC >0.71), and this was improved to AUC 0.77 by combining the expression of two miRNA markers (hsa-miR-374a-5p and hsa-let-7d-5p) indicating their potential use in prediction of SGA.

Previous studies examining miRNAs as potential biomarkers of SGA births have been mainly focused on identifying changes in the expression of placental miRNAs associated with placental dysfunction [32-35]. However, these have limited clinical application. Higasijima et al. showed that FGR pregnancies are characterized by low placental levels of hsa-miR-518b, miR-1323, miR-520 h and miR-519d, but these changes are not reflected in maternal plasma [36]. Similarly Hromadnikova et al. reported no difference in the expression of placental miRNAs, miR-517, miR-510a and miR-525, in maternal plasma of FGR cases and controls [37] despite later showing significantly lower levels of these miRNAs in placental tissue of FGR cases compared to controls [38]. Although the expression of these placental miRNAs were not included in the top 50 most abundant miRNAs detected from our nCounter assay, we did observe differential expression of miR-21−5p, miR-194−5p, miR-574−3p, miR-518b and miR-525−5p in support of previously published data [32-34,36,38] (Supplementary Figure S3a-e). In contrast, placenta-derived miR-141−3p, miR-499a-5p and miR-424−5p, which have been previously reported to be increased in FGR cases [33,35,39]
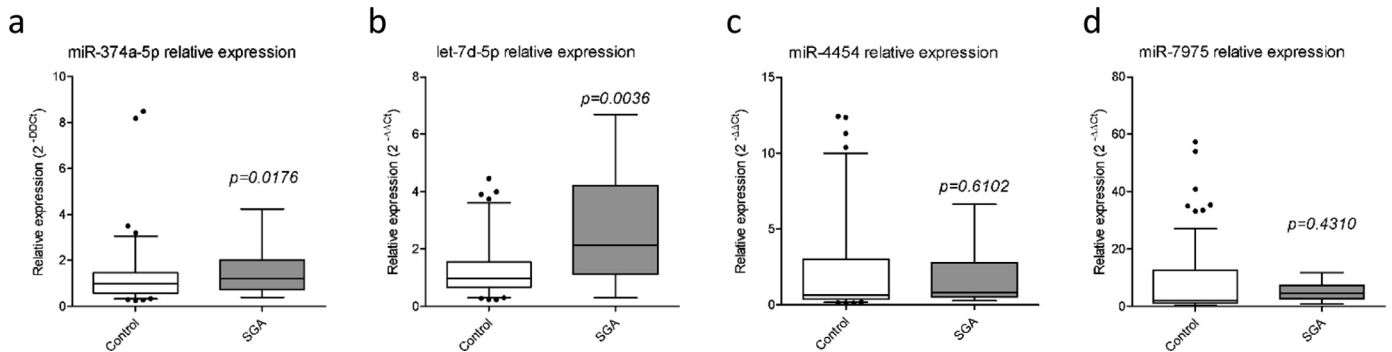

Fig. 5. Expression of technically validated miRNAs in an independent validation cohort at $12^{+0}-14^{+6}$ weeks gestation. Total RNA were extracted from plasma samples and used to validate candidate miRNA expression using RT-qPCR. Resulting Cq values normalized for any discrepancies in the efficacy of RNA extraction using the spike-in cel-254 (5000attomoles) and for reverse transcription using the spike-in UniSp6. Two miRNAs, hsa-miR-30d-5p and hsa-let-7i-5p, identified using NormFinder were used as endogenous controls. All values were then normalized relative to the average of the control samples. The differential plasma levels of **(a)** hsa-miR-374a-5p, **(b)** hsa-let-7d-5p, **(c)** hsa-miR-4454 and **(d)** hsa-miR-7975 were validated using RT-qPCR. P values were determined by unpaired t-test for normally distributed data, and Mann-Whitney U test for non-parametric data (Control n = 83, SGA n = 12). Graphs show median and interquartile range.
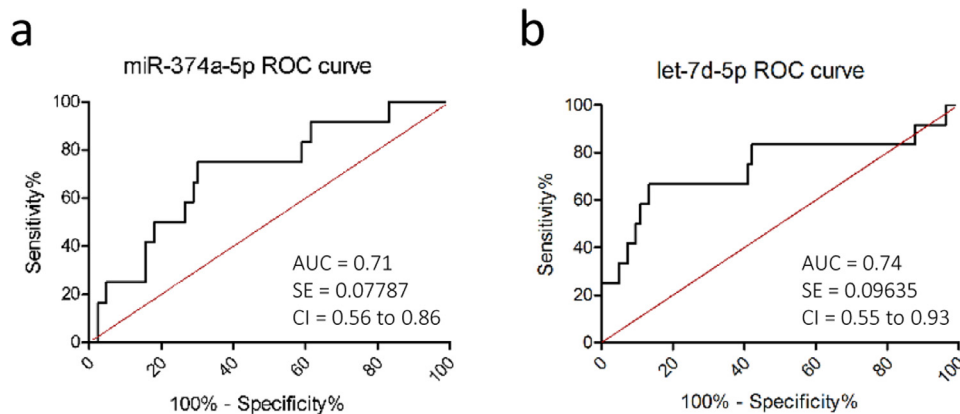

Fig. 6. Comparisons of the predictive ability of individual plasma miRNA markers. ROC analyses were performed using the relative expression of miRNA markers from maternal plasma samples at $12^{+0}-14^{+6}$ weeks gestation, and ROC curves were plotted to determine the predictive values of hsa-miR-374a-5p **(a)**, and hsa-let-7d-5p **(b)** for SGA (Control n = 83, SGA n = 12). AUC: area under the curve, SE: standard error, CI: 95% confidence interval.
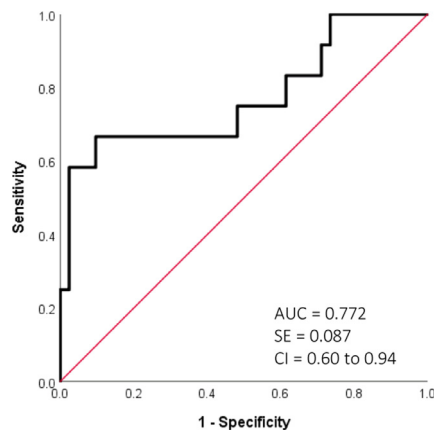
Fig. 7. Predictive ability of combined miRNA markers in maternal plasma ($12^{+0}-14^{+6}$ weeks) for SGA. ROC curve of combination of hsa-miR-374a-5p and hsa-let-7d-5p was plotted to examine their predictive accuracy for SGA. (Control $n = 83$, SGA $n = 12$). AUC: area under the curve, SE: standard error, CI: 95% confidence interval.

were decreased in our SGA cases (Supplementary Figure S3f-h). These results emphasize the high variability in levels of placenta-specific miRNAs reported in the maternal circulation and caution should be used in applying them as biomarkers of placental function until understanding of their release, processing and degradation within the maternal circulation is improved.

Microarray-based techniques have also been recently used to examine the differences in the circulating miRNA expression profiles of SGA (less than 10th centile), average for gestational age (AGA, 10th-90th centile) and large for gestational age (LGA, greater than 90th centile) in maternal serum samples [20]. This study reported lower expression of specific miRNAs including miR-20b-5p, miR-942−5p, miR-324−3p, miR-223−5p, and miR-127−3p to be associated with SGAs. Direct comparison of the expression levels of these miRNAs in our dataset showed that they were all expressed below background levels except for miR-324−3p. This may be due to serum having a higher miRNA expression than plasma as the coagulation process results in additional release of miRNAs from blood cells and platelets [40]. There is an association between maternal platelet count and platelet life span with SGA deliveries, where women with SGA births had a shorter mean platelet life span than women with normal weight births [41,42]. Platelet contribution before and after coagulation results in differences of miRNA composition and platelet-depleted plasma represents a more robust sample for circulating miRNA biomarker discovery.

Our study was limited by its sample size, the lack of information about foetal genetic abnormalities such as sex chromosome abnormalities and mosaicism, and the exclusion of other pregnancy complications. Another limitation of our study design is that the validation cohort was enriched for the primary outcome compared to the general low-risk population, therefore, our ROC curves may overestimate the accuracy of the miRNA markers to predict SGA in the general population. Future large population-wide studies will be needed to validate the performance of these miRNA markers. As no significant changes in the expression of these miRNA markers was observed between 12^{+0} and 21^{+6} weeks gestation, this suggests that these miRNA markers should perform with similar accuracy at any time of the tested gestational windows. For effective clinical interventions however, using earlier samples would be preferable.

Our results highlight unique circulating miRNA expression profiles of SGA, and identify potential candidate plasma miRNAs as predictive markers for SGA births which can be utilised in future bedside tests based on lateral flow assays [43]. The ability to identify those women at risk of SGA early in pregnancy would allow earlier clinical interventions, such as administration of low dose aspirin, focused monitoring of foetal growth, avoidance of drugs/smoking, control of maternal disorders (eg. hypertension), and adjustments to maternal lifestyle and diet, to improve the intrauterine environment and therefore attenuate the causes of impaired growth.

Contributors

D.A.M., P.R.B and V.T. conceived and designed the work; J.C. and L. S. collected the samples; S.H.K. and R.B. performed the experiments; S.H.K. D.A.M. and V.T analysed the data; S.H.K. and D.A.M. prepared the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interests

Dr. Kim reports grants from March of Dimes, during the conduct of the study; In addition, Dr. Kim has a patent miRNA markers for small-for-gestational-age births (SGA) pending. Dr. MacIntyre reports grants from March of Dimes, other from NIHR BRC based at Imperial Healthcare NHS Trust and Imperial College London, during the conduct of the study; In addition, Dr. MacIntyre has a patent miRNA markers for small-for-gestational-age births (SGA) pending. Dr. Binkhamis has nothing to disclose. Dr. Cook has nothing to disclose. Dr. Sykes reports grants from March of Dimes, other from NIHR BRC based at Imperial Healthcare NHS Trust and Imperial College London, during the conduct of the study;. Dr. Bennett reports grants from March of Dimes, other from NIHR BRC based at Imperial Healthcare NHS Trust and Imperial College London, during the conduct of the study; In addition, Dr. Bennett has a patent miRNA markers for small-for-gestational-age births (SGA) pending. Dr. Terzidou reports grants from Genesis Research Trust, grants from March of Dimes, other from NIHR BRC based at Imperial Healthcare NHS Trust and Imperial College London, during the conduct of the study; In addition, Dr. Terzidou has a patent miRNA markers for small-for-gestational-age births (SGA) pending.

Acknowledgements

Data sharing Statement

All de-identified nCounter data are available in Supplementary data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103145.

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