1 Topic: Obstetrics

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3 **Quantifying mRNA coding growth genes in the maternal**
4 **circulation to detect fetal growth restriction**

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30 **Condensation:** RNA coding growth genes in maternal circulation are differentially
31 regulated in severe preterm FGR, and at 28 weeks in pregnancies destined for FGR
32 at term.

33

34 **Short title:** RNA coding growth genes in maternal blood with FGR

35 Abstract

36 **Objective:** To examine whether mRNA circulating in maternal blood coding genes
37 regulating fetal growth are differentially expressed in 1) severe preterm FGR and 2)
38 at 28 weeks' gestation in pregnancies destined to develop FGR at term.

39 **Study Design:** mRNA coding growth genes were measured in two independent
40 cohorts. The first was women diagnosed with severe preterm FGR (<34 weeks
41 gestation; n=20) and gestation matched controls (n=15), where the mRNA was
42 measured in both maternal blood and placenta. The second cohort was a prospective
43 longitudinal study (n=52) of women whom had serial ultrasound assessments of fetal
44 growth. mRNA coding growth genes in maternal blood was measured at 28 and 36
45 weeks in pregnancies with declining growth trajectories (ending up with term FGR;
46 n=10 among the 52 recruited) and controls who maintained normal growth trajectory
47 (n=15).

48 **Results:** In women with severe preterm FGR there was increased expression of
49 PGH (6.3 fold), IGFs (IGF1 3.4 fold, IGF2 5.0 fold), IGF receptors (2.1 fold) and IGF
50 binding proteins (3.0 fold), and reduced expression of ADAM12 (0.5 fold) in maternal
51 blood (and similar trends in placenta) compared to controls ($p<0.05$). Notably, at 28
52 weeks gestation there was increased IGF2 (3.9 fold), PGH (2.7 fold) and IGFBP2
53 (2.1 fold) expression in maternal blood in women destined to develop FGR at term
54 ($p<0.05$).

55 **Conclusion:** Measuring mRNA coding growth genes in maternal blood may detect
56 unsuspected severe preterm FGR already present in utero, and predict term FGR
57 when measured at 28 weeks' gestation.

58
59 **Keywords:** free RNA, maternal circulation, fetal growth restriction, placenta,
60 biomarker.

61

62 **Introduction**

63 Fetal growth restriction (FGR) is a leading cause of stillbirth^{1, 2} and strongly
64 associated with increased perinatal morbidity. There are no effective intrauterine
65 treatments, and therefore the management relies on early detection and timely
66 delivery³.

67 Unfortunately, clinical detection of FGR is suboptimal and many cases remain
68 undiagnosed⁴. It is thought that unrecognized FGR represents an important cause of
69 stillbirth and therefore a reliable screening test for FGR could significantly improve
70 clinical outcomes⁵. There are two situations where a non-invasive biomarker test for
71 FGR may be useful: 1) to identify cases of unsuspected preterm FGR that are
72 already present *in utero*, or 2) predict cases of normally grown fetuses at risk of
73 developing FGR.

74 The discovery that nucleic acids circulate in the maternal blood where they can be
75 quantified offers a novel avenue to identify biomarkers of placental function^{6, 7}. RNA
76 can be extracted reliably and subjected to molecular analyses with precision. In
77 contrast, the detection of proteins relies on the existence of high quality monoclonal
78 antibodies. Importantly, it is possible circulating mRNA in the maternal circulation
79 may be derived from the placenta and reflect trends in differential transcript
80 expression in the distant placental transcriptome^{8, 9}. If true, then this approach could
81 also provide unique insights into placental pathology previously not attainable whilst
82 the fetus remains *in-utero*.

83 The insulin like growth factor (IGF) system is essential for fetal and placental growth
84 and development. It includes IGF1 and IGF2, which can bind to one of six IGF
85 binding proteins (IGFBPs) in order to modulate their bioavailability. The IGFs exert
86 their metabolic actions by interacting with cell surface tyrosine kinase receptors

87 (IGF1R, IGF2R), which selectively bind the IGFs and insulin. Notably, these IGF
88 ligands, receptors and binding proteins are expressed in the placenta, and the IGFs
89 are released into the maternal circulation from early pregnancy¹⁰. Placental growth
90 hormone (PGH) and ADAM12 are recently discovered placental derived growth
91 factors that also modulate their effects via the IGF system. There is mounting
92 evidence that abnormalities in the IGF system play a crucial role in the pathogenesis
93 of FGR. However, the literature has been conflicting as to whether FGR is associated
94 with changes in circulating IGF proteins in maternal blood.

95 Therefore in this study, we investigated whether mRNA of IGF1, IGF2, IGFBP-2,
96 IGF1R, PGH and ADAM12 were dysregulated in the maternal blood in pregnancies
97 affected by severe preterm FGR, and whether the circulating mRNA might reflect
98 placental gene expression. We also studied a second cohort where we prospectively
99 collected serial maternal samples from 28 weeks' gestation in a low risk population
100 and followed fetal growth until delivery. We identified cases with well-grown fetuses
101 at recruitment (as determined by ultrasound) but who exhibited a decline in growth
102 trajectory, developing FGR at term. We measured mRNA coding these six growth
103 factors in maternal blood from this cohort at 28 and 36 weeks' gestation to determine
104 their potential to predict term FGR.

105

106 **Materials and Methods**

107 *Clinical cohorts and recruitment of participants:* Subjects were recruited from Mercy
108 Hospital for Women and Monash Medical Centre, Melbourne between 2008-2011.
109 Written informed consent was obtained and the study protocol was approved by both
110 institutions' Human Research and Ethics Committees.

111 There are two cohorts: a case-control study investigating severe preterm FGR, and a
112 prospective longitudinal study investigating term FGR.

113 In the first cohort, cases of severe preterm FGR were defined as FGR (customized
114 birthweight <10th centile) requiring iatrogenic delivery <34 weeks', with evidence of
115 uteroplacental insufficiency (asymmetrical growth + abnormal umbilical artery
116 Doppler velocimetry, +/- oligohydramnios or abnormal fetal vessel velocimetry). FGR
117 due to infection, chromosomal or congenital abnormalities, and multiple pregnancies
118 were excluded. Both maternal blood and placenta was collected (n=20) and those
119 with or without preeclampsia (ACOG guidelines ¹¹) were included.

120 Preterm control blood samples were collected from women (n=20) with an
121 appropriately grown fetus, at a gestation matched to the preterm FGR cases, and
122 who subsequently delivered appropriately grown fetuses at term without obstetric
123 complications (birthweight 20-80th centile). Preterm placental samples (n=8) were
124 collected from women delivering preterm (<34 weeks) an appropriate grown fetus, in
125 the absence of hypertensive diseases of pregnancy or chorioamnionitis. Term
126 placental samples (n=8) were collected from uncomplicated, appropriately grown
127 (birthweight 20-80th centile) singleton pregnancies at >37 weeks'. All placental
128 samples were collected at pre-labour caesarean section.

129 In the second cohort, low risk healthy pregnant women were recruited at 28 weeks'
 130 gestation. At the time of recruitment, none of the participants had obstetric, medical
 131 or surgical complications. Ultrasound assessment of fetal growth was performed at
 132 28 weeks gestation, and only those with a well-grown fetus were included. Maternal
 133 blood samples were collected at 28 and 36 weeks gestation. Ultrasound assessment
 134 of fetal growth was repeated at 36 weeks gestation and final birthweight recorded.
 135 After birth the subjects were stratified into 3 cohorts (Fig. 3A):

136 1) Appropriate for gestational age fetuses (AGA, n=15), defined as fetuses with an
 137 estimated fetal weight 20-95th centile at 28 weeks who maintained their growth
 138 trajectory to term (change in centiles between the 28 weeks and birth <30%).

139 2) Term FGR (n=10), defined as fetuses appropriately grown at 28 weeks (20-95th
 140 centile) who subsequently exhibited a fall in growth trajectory resulting in term FGR
 141 (serial fall in estimated weight centiles from 28 weeks to 36 weeks gestation, with
 142 birthweight <10th centile)

143 3) The remainder of the pregnancies (n=27), had variable growth trajectories and
 144 could not be clearly classified into either of the above groups. Samples from this
 145 cohort were not analyzed.

146 Estimated fetal weight was calculated using the Hadlock equation¹². The customized
 147 centiles for estimated fetal weight and birthweight were generated using the
 148 Australian dataset of the GROW software (www.gestation.net) which takes into
 149 consideration maternal height, weight, ethnicity, parity and fetal gender¹³. All
 150 ultrasounds were performed by one operator, trained and experienced in fetal
 151 biometry.

152 *Sample collection and laboratory analysis:* Maternal peripheral whole blood samples
 153 (2.5ml) were collected in PAXgene blood RNA tubes (PreAnalytix, Hombrechtikon,

154 Switzerland) and processed as per manufacturer's instructions and stored at -80C
 155 until processing.

156 Placental biopsies were obtained immediately after delivery by caesarean section.
 157 Placental biopsies were taken from the maternal side of the placenta, to a depth of
 158 2/3 of the placenta, avoiding the decidua. The biopsies were clear of obvious
 159 infarction or calcification. Placental biopsies were washed in sterile PBS to remove
 160 contamination by maternal blood, snap frozen and stored at -80C until processing.

161 RNA was extracted from peripheral whole maternal blood using the PAXgene Blood
 162 miRNA kit (PreAnalytix) according to manufacturer's instructions, as previously
 163 described¹⁴. Placental RNA was extracted using the mirVana Isolation Kit (Ambion,
 164 Austin TX) according to manufacturer's instructions. Genomic DNA was removed
 165 using DNase treatment, and total RNA eluted and stored at -80C if not used
 166 immediately. RNA concentration and purity were measured using a NanoDrop
 167 ND1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA).

168 Real time quantitative RT-PCR analysis was performed after reverse transcription of
 169 200ng RNA using Superscript Vilo (Invitrogen, Carlsbad, CA) according to
 170 manufacturers instructions. Quantitative gene expression analyses were performed
 171 with commercially available Taqman Gene Expression Assays (Applied Biosystems,
 172 Carlsbad, CA) for IGF1, IGF2, IGF1R, IGFBP-2, PGH and ADAM12. The RT-PCR
 173 was performed in triplicate, with multiple negative controls (including no template and
 174 no RT controls), on the CFX 384 (BioRad, Foster City, CA), with the following cycling
 175 conditions: 50C for 2 min, 95 C for 10 min, and 40 cycles of 95C for 15 sec, 60C for 1
 176 min, and 72 C for 30 sec.

177 Relative quantification was determined by the comparative CT method. Gene
 178 expression was normalized against the mean expression of GAPDH, GUSB and B2M

179 to increase the stringency of the comparison, and calibrated against the mean
180 expression level of the preterm control group.

181 *Statistical analysis:* All data was statistically analyzed using Graphpad Prism v 5
182 (GraphPad Software Inc., San Diego, CA). Differences in RT-PCR gene expression
183 was assessed using Mann – Whitney U or Kruskal –Wallis test, or where the data
184 was considered normally distributed the t-test or ANOVA. Patient characteristics
185 were compared using χ^2 where appropriate. Data was presented as mean +/- SEM.
186 Significance was defined as $p < 0.05$.

187 Results

188 *mRNA coding growth genes are differentially expressed in placenta from cases of*
 189 *severe preterm FGR.*

190 We first examined the expression of IGF1, IGF2, IGF1R, IGFBP2, PGH and ADAM12
 191 in the placenta in severe preterm FGR (n=20), and compared them with preterm
 192 (n=8) and term (n=8) controls. The clinical characteristics are described in Table 1.

193 In preterm FGR there was a substantial increase in placental mRNA expression of
 194 the following compared to preterm controls (Fig. 1A): IGF1 (2.3 fold, $p<0.05$), IGF2
 195 (3.0 fold, $p<0.01$), IGF1R (2.6 fold, $p<0.01$), IGFBP2 (3.0 fold, $p<0.05$) and PGH (5.6
 196 fold, $p<0.05$). In contrast, ADAM12 was significantly down-regulated in preterm FGR
 197 ($p<0.001$). Interestingly, only IGFBP2 significantly increased across gestation in the
 198 controls, with a 3-fold increase in term controls compared to preterm controls
 199 ($p<0.05$).

200 *mRNA coding growth genes in maternal blood are differentially expressed in cases of*
 201 *severe preterm FGR, and mirror trends seen in the placenta.*

202 To investigate whether circulating mRNA in the maternal circulation might reflect
 203 changes in the placental transcriptome, the expression of these six genes were
 204 measured in maternal blood (Fig. 1B). The expression of all six genes in maternal
 205 blood with severe preterm FGR closely correlated with the trends in the placental
 206 transcriptome (compared with Fig. 1A). Interestingly, the degree of fold change was
 207 generally higher in maternal blood compared to placenta. Thus, in preterm FGR there
 208 was a 3.4 fold increase in IGF1 ($p<0.001$), 5.0 fold increase in IGF2 ($p<0.01$), 2.1 fold
 209 increase in IGF1R ($P<0.05$), 6.3 fold increase in PGH ($p<0.01$) and 3.0 fold increase
 210 in IGFBP2. Like placenta, ADAM12 expression was down-regulated in the maternal
 211 blood in preterm FGR compared to controls ($p<0.05$).

212 *Expression mRNA coding growth genes in maternal blood correlates with the severity*
 213 *of disease, in severe preterm FGR.*

214 The severity of preterm FGR is characterized by increased placental resistance,
 215 which can be assessed by Doppler ultrasound of placental and fetal vessels. The
 216 mildest abnormality is reflected by an increase in the umbilical artery resistance
 217 (pulsatility index), whereas absent and reversal of end diastolic flow in the umbilical
 218 artery (AREDF) is associated around 30-70% destruction of the placental villous tree,
 219 and strongly correlated with fetal hypoxia/acidemia¹⁵. We therefore examined
 220 expression of mRNA coding growth genes in maternal circulation was further
 221 dysregulated with increasing severity of FGR.

222 Fig. 2 demonstrates increased expression in both the placenta (Fig. 2A) and
 223 maternal blood (Fig. 2B) for IGF1, IGF2 and PGH with AREDF compared to
 224 increased resistance but positive end diastolic flow (Increased PI; $p < 0.05$). Again,
 225 the fold changes in the maternal blood were far greater than that seen in placenta.
 226 There was a 2-fold increase in IGF1 in the placenta compared to a 4.8 fold increase
 227 in the maternal blood with AREDF. However, there was no difference in expression
 228 of IGFBP2, IGF1R or ADAM2 in either the placenta or maternal blood in FGR,
 229 stratified according to the severity of umbilical artery Doppler resistance.

230 *mRNA coding growth genes in maternal blood gestation are differentially expressed*
 231 *at 28 and 36 weeks' in pregnancies destined to develop term FGR.*

232 We next investigated whether mRNA coding growth genes in the maternal circulation
 233 at mid-pregnancy can identify fetuses that are appropriately grown at 28 weeks
 234 gestation, but develop late placental insufficiency evidenced by a decreased growth
 235 trajectory, and term FGR.

236 We recruited 52 healthy pregnant women at 28 weeks, performing an ultrasound to
 237 confirm apparent normal fetal growth (EFW >20th centile) at the time of recruitment.
 238 We then followed participants longitudinally, collecting maternal blood at 28 and 36
 239 weeks gestation and tracking growth trajectory by performing another ultrasound at
 240 36 weeks, then noting final birthweight (Fig. 3 depicts how participants were
 241 recruited).

242 Of the 52 participants, we identified 15 appropriate grown controls (AGA) where the
 243 fetus clearly maintained growth trajectory (Fig. 3B is an example of a clinical growth
 244 chart plot where fetal growth was maintained) and 10 cases where growth trajectory
 245 serially declined and the pregnancy ended up with FGR at term (birthweight <10th
 246 centile; Fig. 3C). Clinical characteristics of this second cohort are shown in Table 2.
 247 None of the individuals in the second cohort developed pre-eclampsia. We then
 248 compared the mRNA coding growth transcripts in the maternal blood among the AGA
 249 controls and term FGR cases (Fig. 4).

250 Firstly we examined the expression of the growth transcripts across gestation in the
 251 AGA controls. There was a non-significant increase in the expression of IGF1, IGF2
 252 and PGH between 28 and 36 weeks, but no change across gestation in IGFBP2,
 253 IGF1R and ADAM12.

254 We next examined whether the expression of the growth transcripts in maternal
 255 blood was different between AGA and term FGR. There was a 2.2 fold increase in
 256 IGF1 and IGFBP2 ($p<0.05$), a 2.7 fold increase in IGF2 ($p<0.01$) and a 3.0 fold
 257 increase in PGH ($p<0.01$), in pregnancies on the way to developing FGR at term, but
 258 with an estimated fetal weight still above the 10th centile on ultrasound at 36 weeks
 259 (Fig. 4A). If these women had only had an ultrasound assessment of fetal size at 36
 260 weeks, it is unlikely term FGR would have been predicted. In contrast, in cases with
 261 evolving term FGR, there was already evidence of differential expression of mRNA

262 coding growth genes at 36 weeks. This suggests a blood test performed at 36 weeks'
263 gestation may potentially identify more cases of term FGR than an ultrasound at the
264 same gestation.

265 Finally, we examined the expression of the growth transcripts in maternal blood at 28
266 weeks gestation. We confirmed on ultrasound that all fetuses were well grown at the
267 time of blood sampling and there were no risk factors for FGR. In contrast, the
268 expression of IGF2 (3.9 fold, $p<0.01$), PGH (2.7 fold, $p<0.05$) and IGFBP2 (2.1 fold,
269 $p<0.05$) were already significantly increased in pregnancies that would subsequently
270 develop FGR at term (Figure 4B). Thus these transcripts were altered more than 10
271 weeks prior to the clinical manifestation of FGR.

272

273 **Comment**

274 FGR is strongly associated with severe perinatal morbidity and mortality, possibly
 275 accounting for up to 50% of unexplained stillbirths^{1,2}. Infants with FGR that survive
 276 the neonatal period are also at risk of delayed childhood neurodevelopment including
 277 cerebral palsy, as well as adult metabolic and cardiovascular disease¹⁶.
 278 Unfortunately, there are no effective intrauterine treatments for FGR, and the goal of
 279 management is early detection and timely delivery³. Current methods of clinical
 280 detection of the growth-restricted fetus have poor sensitivity and specificity. Some
 281 have estimated as many as 75-85% of FGR cases are missed antenatally^{4, 17}.
 282 Furthermore, there are no good cost effective biomarkers for FGR that can be
 283 applied to a low risk population to either predict FGR or identify FGR already present
 284 *in utero*.

285 We have investigated levels of mRNA circulating in maternal blood that code major
 286 growth genes in pregnancy using two approaches: detection of severe preterm FGR
 287 already present *in-utero*, and early prediction of FGR at term. In the severe preterm
 288 FGR study, we found mRNA expression of six growth factors in the maternal blood
 289 were dysregulated with severe preterm FGR, and mirrored trends seen in the
 290 placental transcriptome. In addition, they were further dysregulated with increasing
 291 severity of disease as determined by umbilical artery Doppler velocimetry. In our
 292 second study, we recruited low risk women at 28 weeks and tracked fetal growth
 293 longitudinally, and found mRNA coding growth genes in maternal circulation were
 294 dysregulated as early as 28 weeks gestation in those women who subsequently
 295 developed FGR. Therefore circulating growth factor RNA in the maternal blood may
 296 be a promising method to both detect established preterm FGR and predict FGR at
 297 term.

298 Interestingly, the trends in mRNA expression of all six growth genes in maternal
 299 blood not only mirrored those seen in placenta but the fold changes were generally
 300 much greater in maternal blood. We hypothesize the reason is that the origin of most
 301 of the circulating mRNA is shed particles from the syncytiotrophoblast layer, the
 302 same tissue layer that expresses the growth transcripts¹⁸. As such, mRNA in
 303 maternal blood might be naturally enriched with IGF mRNA transcripts from the
 304 syncytiotrophoblast. If correct, this lends support to the promise of exploiting free
 305 circulating RNA of placental origin as a clinical biomarker.

306 Importantly, a strength of our study is the inclusion of both severe preterm FGR
 307 cases and term FGR, diseases that are managed very differently in the clinic.
 308 Preterm FGR occurs less frequently but has a strong association with perinatal
 309 mortality and neonatal morbidity due to prematurity. Therefore identifying these
 310 pregnancies to optimize timing of delivery is clinically important⁵. Term FGR is a
 311 substantial contributor to perinatal mortality and notably, the incidence of stillbirth due
 312 to FGR is at its peak after 38 weeks. Therefore, induction of labour in these
 313 pregnancies is likely to yield the greatest clinical benefit with minimal harm.

314 Routine ultrasound applied to low risk pregnancies has not been shown to improve
 315 perinatal outcome¹⁹. In our longitudinal study, we have obtained encouraging
 316 evidence that measuring mRNA coding growth genes at 28 weeks gestation, even
 317 with a normal fetal size on ultrasound at the time of the blood test, may identify
 318 pregnancies at risk of term FGR. Furthermore, in our study, a number of these
 319 babies would have been missed by ultrasound at 36 weeks. This is of relevance
 320 clinically given an ultrasound is often timed at 36 weeks in suspected cases of FGR
 321 occurring near term. In contrast, the changes in mRNA expression in maternal blood
 322 in those destined to develop term FGR were not only still present at 36 weeks
 323 gestation, but more marked. While our findings in this longitudinal study are highly
 324 encouraging, they require validation in a larger prospective study.

325 In conclusion, we have confirmed that the expression of RNA transcripts for growth
326 factors, circulating in the maternal blood may be novel predictive and diagnostic
327 biomarkers for FGR. A predictive and diagnostic test for FGR would enable clinicians
328 to identify pregnancies that would benefit from intensive monitoring and early delivery
329 to reduce the morbidity, and potentially the rates of stillbirth, due to undiagnosed
330 FGR.

331

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385

386

387 **Table 1.** Clinical characteristics of patients in the severe preterm FGR study

		FGR Blood and placenta (n= 20)	Preterm Blood (n=20)	Preterm Placenta (n=8)	Term Placenta (n=8)
Maternal age (yrs)		30.4 (5.9)	31.1 (1.5)	31.8 (1.7)	31.9 (1.1)
Parity (% primiparous)		65	60	12.5	25
Gestational age at delivery (weeks)		29+5 (3)	40+2 (1.4)	29+4 (1)	38+3 (0.6)
Gestational age at sampling (weeks)		29+5 (3)	30+1 (2.1)	29+4 (1)	38 +3 (0.6)
Birthweight (gms)		885 (273)	3565 (35)	1492 (203)	3400 (122)
Customised birth centile (%)		3 (3)	48 (6)	66 (7.5)	69 (4)
Perinatal death (%)		13	0	0	0
Pre-eclampsia (%)		40	0	0	0
Umbilical artery Doppler velocimetry waveforms	REDF	30	0	0	0
	AEDF	40	0	0	0
	↑ PI	30	0	0	0
	Normal (%)	0	100	100	100

388 Data is presented as mean (SEM) or %.

389 REDF: reverse end diastolic flow. AEDF: absent end diastolic flow. ↑ PI: increased

390 pulsatility index.

391

392 **Table 2.** Clinical characteristics of patients in the longitudinal term FGR study

		Appropriately grown at term (n=15)	Term FGR (n=10)
Maternal age (yrs)		32.0 (1.2)	31.1 (1.8)
Parity (% primiparous)		27	60
Mode of delivery (%)	Operative	40	60
	SVD	60	40
Gestational age at 1 st USS/ blood sampling (weeks)		28+1 (0.1)	28+1(0.1)
Gestational age at 2 nd USS/ blood sampling (weeks)		35+4 (0.1)	35+3(0.1)
Gestational age at delivery		39+0 (0.3)	39+0(0.4)
Estimated fetal weight at 28 weeks (gms)		1266 (50)	1200 (42)
Customised centile at 28 weeks (%)		61(7)	51 (8)
Estimated fetal weight at 36 weeks (gms)		2839 (83)	2420 (60) **
Customised centile at 28 weeks (%)		57 (7)	28 (4) **
Birthweight (gms)		3580 (85)	2780 (120)***
Customised birth centile (%)		56 (6)	9 (2) ***

393 Data is presented as mean (SEM) or %. **p<0.01, *** p<0.001

394

395 **Figure Legends**

396 **Fig. 1.** mRNA expression of IGF1, IGF2, IGF1R, IGFBP2, placental GH and
 397 ADAM12 in placenta (A) and maternal blood (B) in severe preterm FGR compared to
 398 well grown preterm and term controls (A only for term controls). Normalized against
 399 GAPDH, GUSB and B2M. Data is presented as median +/- IQR. Mann-Whitney test
 400 was used for statistical comparison to preterm controls. * $p < 0.05$, ** $p < 0.01$,
 401 *** $p < 0.001$.

402

403 **Fig. 2.** Expression of IGF1, IGF2 and placental GH in placenta (A) and maternal
 404 blood (B) in severe preterm FGR stratified according to the severity of umbilical
 405 artery Doppler velocimetry. SDR: raised diastolic systolic ratio, positive end diastolic
 406 flow. AREDF: absent or reversed end diastolic flow. Normalized against GAPDH,
 407 GUSB and B2M. Data is presented as median +/- IQR. Mann-Whitney test was used
 408 for statistical comparison to preterm controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

409

410 **Fig. 3.** Approach to select case and controls for the longitudinal study to predict term
 411 FGR. The control group (AGA) consisted of pregnancies where the fetus maintained
 412 its growth trajectory from 28 weeks to birth and remained well grown. The cases
 413 (term FGR,) consisted of pregnancies where the fetus was initially well grown at 28
 414 weeks but failed to maintain its growth trajectory, with a birthweight less than 10th
 415 centile. (B) is a growth chart plot, and depicts an example of a case (from AGA
 416 group) where the fetus maintained growth trajectory where (C) is an example where
 417 the fetus did not maintain growth trajectory and ended up as term FGR. Customised
 418 growth charts generated from GROW software (www.gestation.net) for example case
 419 and control.

420

421 **Fig. 4.** RNA expression of IGF1, IGF2, placental GH and IGFBP2 in maternal blood
422 at (A) 36 weeks' gestation and (B) 28 weeks gestation in women who subsequently
423 delivered a baby with term FGR, compared to controls (cases where the fetus
424 maintained growth trajectory). Normalized against GAPDH, GUSB and B2M. Data is
425 presented as median +/- IQR. Mann-Whitney test was used for statistical comparison
426 to preterm controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
427

Figure 1

A: Placenta

B: Maternal blood

Figure 2

A: Placenta

B: Maternal blood

Figure 3

Figure 4
A: 36 weeks

B: 28 weeks

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