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Changes in the Metabolic Footprint of Placental Explant-Conditioned Medium Cultured in Different Oxygen Tensions from Placentas of Small for Gestational Age and Normal Pregnancies

R.P. Horgan [a,*], D.I. Broadhurst [a], W.B. Dunn [b,c,d], M. Brown [e], A.E.P. Heazell [f], D.B. Kell [b,c], P.N. Baker [g], L.C. Kenny [a]

[a] The Anu Research Centre, Department of Obstetrics and Gynaecology, University College Cork, Cork University Maternity Hospital, Cork, Ireland
[b] The Manchester Centre for Integrative Systems Biology, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK
[c] School of Chemistry, Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK
[d] Centre for Advanced Discovery and Experimental Therapeutics, Manchester Biomedical Research Centre, University of Manchester, Manchester, UK
[e] The Manchester Biomedical Research Centre, Manchester Royal Infirmary and University of Manchester, UK
[f] The Maternal and Fetal Health Research Centre, The University of Manchester, St Mary's Hospital, Whitworth Park, Manchester M13 0JH, UK
[g] Faculty of Medicine & Dentistry, University of Alberta, 2J2.01 WMC, 8440 – 112 Street, Edmonton, AB T6G 2R7 Canada

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ABSTRACT

Being born small for gestational age (SGA) confers significantly increased risks of perinatal morbidity and mortality. Accumulating evidence suggests that an SGA fetus results from a poorly perfused and abnormally developed placenta. Some of the placental features seen in SGA, such as abnormal cell turnover and impaired nutrient transport, can be reproduced by culture of placental explants in hypoxic conditions. Metabolic footprinting offers a hypothesis-generating strategy to investigate factors absorbed by and released from this tissue *in vitro*. Previously, metabolic footprinting of the conditioned culture media has identified differences in placental explants cultured under normoxic and hypoxic conditions and between normal pregnancies and those complicated by pre-eclampsia. In this study we aimed to examine the differences in the metabolic footprint of placental villous explants cultured at different oxygen (O_2) tensions between women who deliver an SGA baby ($n = 9$) and those from normal controls ($n = 8$). Placental villous explants from cases and controls were cultured for 96 h in 1% (hypoxic), 6% (normoxic) and 20% (hyperoxic) O_2. Metabolic footprints were analysed by Ultra Performance Liquid Chromatography coupled to an electrospray hybrid LTQ-Orbitrap Mass Spectrometry (UPLC-MS). 574 metabolite features showed significant difference between SGA and normal at one or more of the oxygen tensions. SGA explant media cultured under hypoxic conditions was observed, on a univariate level, to exhibit the same metabolic signature as controls cultured under normoxic conditions in 49% of the metabolites of interest, suggesting that SGA tissue is acclimatised to hypoxic conditions *in vivo*. No such behaviour was observed under hyperoxic culture conditions. Glycerophospholipid and tryptophan metabolism were highlighted as areas of particular interest.

1. Introduction

Being born small for gestational age (SGA) confers significantly increased risks of perinatal morbidity and mortality and increases the possibility of hypertension and metabolic disorders in later life [1–4]. Depending on the definition used, up to 10% of all live-born neonates are designated as SGA. Although some SGA infants are constitutionally small without adverse risk, a significant proportion of SGA infants have fetal growth restriction (FGR), a condition characterised by a failure of the fetus to achieve its genetically determined size [5,6]. As failure to achieve optimum fetal growth potential is complex to define and measure, the term SGA is used as an alternative. Use of individualised birthweight centiles, which are used in this study, excludes more babies that are constitutionally small, focussing on infants who have "pathological SGA" who at risk of perinatal morbidity and mortality [7].

SGA has a diverse aetiology, which makes elucidation of its pathophysiology challenging. Most research has focused on the role of uteroplacental insufficiency which results in a compromised supply of nutrients and oxygen to the fetus. Accumulating evidence

* Corresponding author. Tel.: +353 21 4205023; fax: +353 21 4205025.
E-mail address: richard.horgan@ucc.ie (R.P. Horgan).

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suggests that SGA may result from complex biochemical interactions within a poorly perfused placenta [8,9] or reduced placental transport of nutrients [10,11]. Compared to those from pregnancies resulting in normal birthweight infants, placentas from women who deliver SGA infants have macroscopic evidence of infarction and microscopic changes including increased formation of syncytial knots, reduced cytotrophoblast proliferation and increased apoptosis [12,13].

Changes in normal oxygen tensions in the placenta have been implicated in the pathophysiology of FGR. Oxygen (O_2) tension is a key regulator of cytotrophoblast proliferation and differentiation [14,15] and therefore on placental growth. Placental hypoxia is hypothesized to be involved in the pathophysiology of SGA as abnormal uteroplacental blood flow and/or umbilical-placental blood flow may result in a reduction of O_2 (and nutrients) available for fetal use [16]. In support of this, pregnancies in women living at high altitude, and therefore relative placenta hypoxia, have an increased incidence of SGA accompanied by expected histological features [17]. Several authors have shown that reduced oxygenation *in vitro* replicates some features of SGA placentas including increased apoptosis and necrosis, reduced cytotrophoblast proliferation and increased syncytial knots [14,15,18–20]. Increasing O_2 tension also alters placental cell turnover, with exaggerated cytotrophoblast proliferation and syncytium formation and increased formation of syncytial knots in placental villous explants [18,19].

There is a wide variation in the delivery of O_2 to the intervillous space in normal pregnancy and the effect of O_2 is dependent on the gestational age, the type of trophoblast and the type of tissue culture under investigation [14,15,21]. At term 6–8% O_2 represents normoxic and 1–2% hypoxic conditions for villous tissue at term [22]. In this study 20% O_2 was used as hyperoxic conditions as in previous studies [14,18,19,23,24].

Metabolic footprinting [25–27] is a powerful hypothesis-generating strategy for investigating the low molecular weight (bio) chemical factors (metabolites) absorbed by and released from a cell culture or tissue *in vitro* [28,29]. Hence, the conditioned culture medium is studied. The position of the metabolome as the final downstream product of gene expression provides a high-resolution multi-factorial phenotypic signature of disease aetiology, manifestation or pathophysiology [30,31].

Metabolomic strategies have been applied to study hypoxia and anoxia in placental tissue from normal and pre-eclamptic pregnancies and those from high altitude. Tissot van Patot et al. demonstrated metabolic differences in placental tissue from pregnancies at low and high altitude using nuclear magnetic resonance (NMR) technology [32]. Studies employing metabolic footprinting using gas chromatography–mass spectrometry showed changes in the concentration of redox metabolites at different oxygen tensions (1, 6 and 20%) [23]. A separate study has shown metabolic differences between placental explants cultured at 1% and 6% O_2 in normal pregnancies and women with pre-eclampsia using Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC–MS) [33].

We proposed to use this state of the art metabolomics technology (UPLC–MS) to examine changes in the metabolic footprints of placental villous explants cultured in different O_2 tensions (1, 6 and 20%) from placentas of small for gestational age and those from normal pregnancies. We hypothesized that samples from normal pregnancies would show their normal *in vivo* metabolic signature at 6% O_2 whilst the SGA samples would reflect their normal *in vivo* metabolic signature at something less than 6%. In addition, reduction of O_2 tension to 1% should render the control samples more like the SGA, whilst 6% O_2 for the SGA samples might result in changes consistent with an increased O_2 tension (20%) in the control.

2. Methods and materials

2.1. Culture of placental villous explants

Placentas were obtained with written consent in compliance with local ethics committee approval (Central Manchester Research Ethics Committee, 08/H1008/22). Placentas were collected from women with uncomplicated term pregnancies resulting in delivery of a healthy singleton fetus ($n = 8$) and from women with suspected SGA which was subsequently confirmed after delivery based upon individualised birthweight centile scores less than the 5th percentile ($n = 9$) [34] (www.gestation.net). Pregnancies complicated by any other maternal or fetal factor, including pre-eclampsia, diabetes mellitus and congenital anomalies were excluded.

Placentas (all received within 20 min of delivery) were sampled at three central placental lobules. Central sampling was chosen as it was deemed the most representative and reproducible location to reflect optimal placental function [35]. Nine explants (3 sets of 3) were cut from three separate placental lobules (27 explants from each placenta), and subsequently stratified so that 3 explants sets (one from each lobule) were cultured at each O_2 tension (1%, 6% and 20%). In total, 153 metabolic footprint samples were collected.

Placental villous explants weighing approximately 5 mg per fragment were prepared as previously described [18] and cultured at the gas–medium interface of fetal bovine serum supplemented with CMRL-1066 medium derived from a single batch for 48 h at 37 °C in an atmospheric O_2 tension of either 1%, 6% or 20%. After 48 h, culture medium was replaced with fresh medium equilibrated to the appropriate O_2 level for 24 h. The explants cultured at 1% O_2 tension remained within the hypoxic chamber when cultured medium was changed. Following a further 48 h conditioned culture medium was collected and immediately frozen at −80 °C.

2.2. Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC–LTQ/Orbitrap) analysis

Metabolic footprint samples were prepared as previously described by thawing on ice of the conditioned culture medium followed by lyophilisation of a 100 μl aliquot [33]. All media samples were analysed in a random order after reconstitution in 200 μl of water using Ultra Performance Liquid Chromatography–Mass Spectrometry (Waters ACQUITY UPLC system coupled to a ThermoFisher Scientific electrospray hybrid LTQ-Orbitrap mass spectrometer). Instrument methods applied here have been previously described in Ref. [33]. Raw centroid data was deconvolved into a peak table using XCMS software v4.1 to report 1676 features (described by a retention time and accurate mass). The data is not quantitative as calibration curves for each detected feature have not been experimentally acquired. The data is reported as responses, calculated as the chromatographic peak area. The term *metabolic signature* is used to reflect the resulting changes elicited by specific conditions. Full details of all methods pertaining to sample preparation and UPLC–MS analysis are described in the attached Supplementary methodology file.

2.3. Statistical analysis

A combination of univariate and multivariate statistical analysis were performed. Supervised multivariate analysis was employed in the form of Canonical Variates Analysis (CVA) [36] combined with Principal Components Analysis (PCCVA), in order to look at system-wide multivariate changes in the metabolic footprint. The number of Principal Components used in the discriminant model was optimized by employing 0.632 + Bootstrap substitution [37], using 100 bootstrap samples to produce an unbiased estimate of prediction. All data were normalised to zero mean and unit variance before performing any multivariate statistics in order to give equal weighting to all metabolite features.

Univariate statistical analysis was used to mine the data further once global changes were characterised. Metabolite features were tested using either the Mann–Whitney test or Student's *t*-test, depending on data normality. The critical *p*-value for significance was set to 0.05. No correction for multiple comparisons was performed as the univariate analysis mainly consisted in a comparison between the different experimental conditions. However, False Discovery Rate (FDR) [38] analysis was performed on all the statistical comparisons, and FDRs quoted where appropriate. All identified metabolites are quoted with both *p*-values and FDR corrected *q*-values.

2.4. Metabolite identification

Metabolites were putatively identified by the matching of the measured accurate mass to accurate mass(es) present in the Manchester Metabolomics Database (MMD) [39]. Where further retention time data was available in mass spectral libraries developed in Manchester, a definitive identification was performed. Definitive identifications are described with an asterisk (*) in Table 2. Using UPLC–MS, a single metabolite is often detected as multiple chromatographic peaks of the same retention time and different accurate mass. This is due to chemical adduction, dimerization, multiple-charging, isotope peaks and fragmentation. In this paper the term *metabolite feature* refers to any detected chromatographic peak

and *metabolite* refers to a 'unique' identified metabolite. For further details please see the attached Supplementary methodology file.

3. Results

Maternal characteristics and pregnancy outcomes of the cases and controls are described in Table 1. Age, BMI, parity, smoking and baby sex were carefully matched across cases and controls. All SGA babies had an individualised birthweight centile ≤5th centile, six were <3rd centile. Eight of the nine SGA babies had oligohydramnios. All control babies were term deliveries with normal liquor volume. One case of SGA was delivered prior to 37 weeks' gestation.

3.1. Global changes in the metabolic footprint of conditioned culture medium from different O_2 tensions

UPLC–MS analysis reproducibly detected a total of 1676 metabolite features. A cross-validated multivariate discriminant model (PC-CVA) was built using all the detected features. The resulting scores plot (Fig. 1) demonstrated differences between the six defined sample groups ($control_{1\%}$; $SGA_{1\%}$; $control_{6\%}$; $SGA_{6\%}$; $control_{20\%}$; $SGA_{20\%}$). The primary change in metabolic profile (CV1) reflected the change in O_2 tension. A secondary difference between SGA and control classes at each O_2 tension was also detected (CV2). The 90% confidence circles show that there was a significant difference in the multivariate means between the SGA and control classes at 6% and 20% O_2; however, not at 1%, although there was a slight difference in populations this difference was not significant.

Further investigation showed that the discrimination seen in CV1 was due to a systemic increase in relative (to 6% O_2 tension) metabolite concentration as the O_2 tension was reduced and a systemic decrease in relative metabolite concentration as the O_2 tension was increased.

3.2. Characterising the differences between the metabolic footprint of conditioned culture medium from SGA and control pregnancies at each O_2 tension

Univariate hypothesis testing was performed across the 1676 detected metabolite features. A total of 574 metabolite features showed significant difference between SGA and normal at one or more of the oxygen tensions. Fig. 2 shows a Venn diagram showing the distribution of the significant metabolite features at each oxygen tension. Of the 574 features that were significantly different at one or more of the oxygen tensions, 221 were identified as 'unique' endogenous metabolites, after removing the multiple matches of, for example, chemical adducts and isotope peaks [39]. These metabolites are listed in full in Supplementary file. 16 metabolites were significant ($p < 0.05$) at all oxygen tensions, 12 metabolites at both 1% and 6% O_2, but not 20%, and 45 metabolites at both 6% and 20% O_2, but not 1%.

95% of the 574 metabolite features that showed significant difference between SGA and normal at any O_2 tension showed a lower mean metabolite level in the SGA samples when compared to the controls.

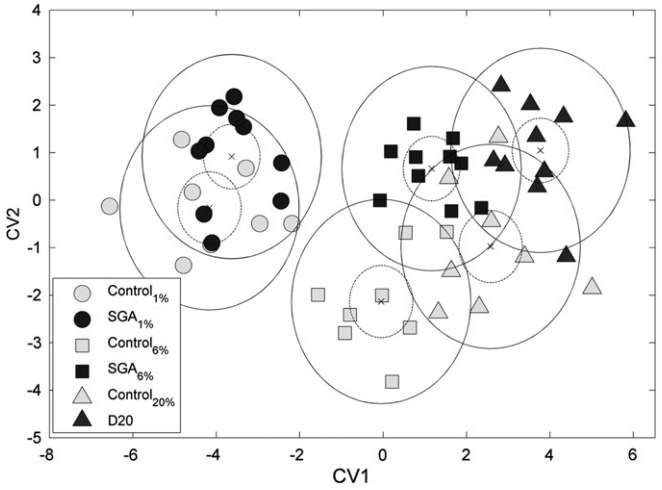

Fig. 1. The figure shows the PC-CVA scores plot (using all the detected metabolite features) for a model built and cross-validated using the first 20 PC scores to discriminate between the six medium classes of interest. Circles represent 1% oxygen samples, squares represent 6% oxygen samples and triangles represent 20% oxygen samples. Open shapes represent control samples and closed shapes represent SGA samples at each oxygen tension. For clarity the placental biological replicates have been averaged. The major change in metabolic footprint is due to oxygen tension (CV1). CV2 reflects a change in footprint reflecting SGA phenotype across all O_2 tensions. The units of the *x*- and *y*-axis are arbitrary due to normalisation of the data. CV = canonical variate; PC-CVA = Principal Components-Canonical Variates Analysis; SGA = small for gestational age; inner circle = 90% CI of the population mean; outer circle = 90% CI of the population itself.

3.3. Relative changes in the normoxic metabolic footprint due to culturing at 1% and 20% O_2 tensions

The data were reduced to the 427 metabolite features that showed significant difference between SGA and control samples cultured at

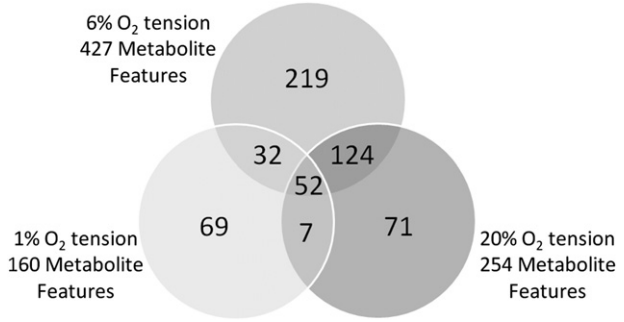

Fig. 2. Venn diagram showing the distribution of the significant metabolite features at each oxygen tension. A total of 574 metabolite features showed significant difference between SGA and normal controls at one or more of the oxygen tensions ($p < 0.05$). At 6% O_2 427 metabolite features were significantly different with a False Discovery Rate of 0.1. At 1% O_2, 160 metabolite features were significantly different (FDR = 0.4). At 20% O_2, 254 metabolite features were significantly different (FDR = 0.2). Of the 574 metabolite features, 52 metabolite features were significantly different at all oxygen tensions (9% of the 574 metabolites); 32 metabolite features were significant at both 1% and 6% O_2, but not 20% (6% of the 574); 124 were significant at both 6% and 20% O_2, but not 1% (22%); 7 were significant at both 1% and 20% O_2, but not 6% (1%).

Table 1
Maternal characteristics and pregnancy outcomes.

Parameter	Control (range)	SGA (range)	*t*-test
Age	29 (18–35)	28 (20–35)	NS
Parity –nullip	6/8	7/9	NS
BMI Kg/m^2	22.9 (19.5–25.5)	23.2 (20.2–25.9)	NS
Smoker –yes	2/8	2/9	NS
Sex –male	5/8	5/9	NS
Birthweight (g)	3376 (2810–4655)	2253 (769–2840)	0.002
Mode of Delivery	4	3	NS
–vaginal	4	6	NS
–C/S			
Gestation	39 + 1 (37+2 – 40 + 4)	38 + 3 (29+5 – 41 + 4)	NS

The table shows the maternal characteristics and pregnancy outcomes of the SGA ($n = 9$) and control ($n = 8$) babies. BMI = body mass index; C/S = caesarean section.

6% O_2 (normoxic conditions). 161 'unique' endogenous metabolites have been putatively identified from these 427 features. For clarity, the footprint for this group of metabolites will be identified as the '*normoxic biomarker signature of SGA*' for the remainder of the paper.

In order to characterise exactly how the metabolic footprint for each sample type differed when cultured at different O_2 tensions, a set of paired two-sample *t*-tests was performed. This was possible as explants from the *same* placenta were cultured at the different O_2 tensions allowing pair-wise matching. For each metabolite of interest, the following four comparison groups were tested: $SGA_{6\%v1\%}$ (paired *t*-test between the SGA explants cultured at 6% O_2 and the SGA explants cultured at 1% O_2) $control_{6\%v1\%}$, $SGA_{6\%v20\%}$ and $control_{6\%v20\%}$. Fig. 3(a) compares the significant changes in footprint due to changing oxygen culture conditions from 6% to 1% O_2 ($SGA_{6\%v1\%}$ vs. $control_{6\%v1\%}$), while Fig. 3b compares the significant changes due to changing from 6% to 20% O_2 ($SGA_{6\%v20\%}$ vs. $control_{6\%v20\%}$).

3.3.1. Changes in relative metabolite levels when culture conditions were changed from 6% to 1% O_2

When comparing the change in metabolite levels after decreasing the O_2 tension from 6% to 1% O_2 (normoxia to hypoxia) four general trends were observed (Fig. 3a):

(i) 79 metabolites showed a significant increase in SGA sample concentration but no significant change in the controls (triangles; 49%) (Table 2).
(ii) 16 metabolites showed a significant decrease in control sample concentration but no significant change in SGA (squares; 10%) (Table 3).
(iii) 17 metabolites showed significant increase in both SGA and control samples (circles; 10%).
(iv) 44 metabolites showed no significant change in either the SGA or control samples (crosses; 27%).

3.3.2. Changes in relative metabolite level when culture conditions were changed from 6% to 20% O_2

When comparing the change in metabolite levels due to increasing the oxygen tension from 6% to 20% O_2 three trends were observed (Fig. 3b):

(i) 28 metabolites showed a significant decrease in control sample concentration but no significant change in SGA (squares; 17%).
(ii) 78 metabolites showed a significant decrease in both SGA and control samples (circles; 48%).
(iii) 44 metabolites showed no significant change in either the SGA or control samples (crosses; 27%).

Only 3 metabolites showed a significant increase in SGA sample concentration but no significant change in the controls (triangles; 2%).

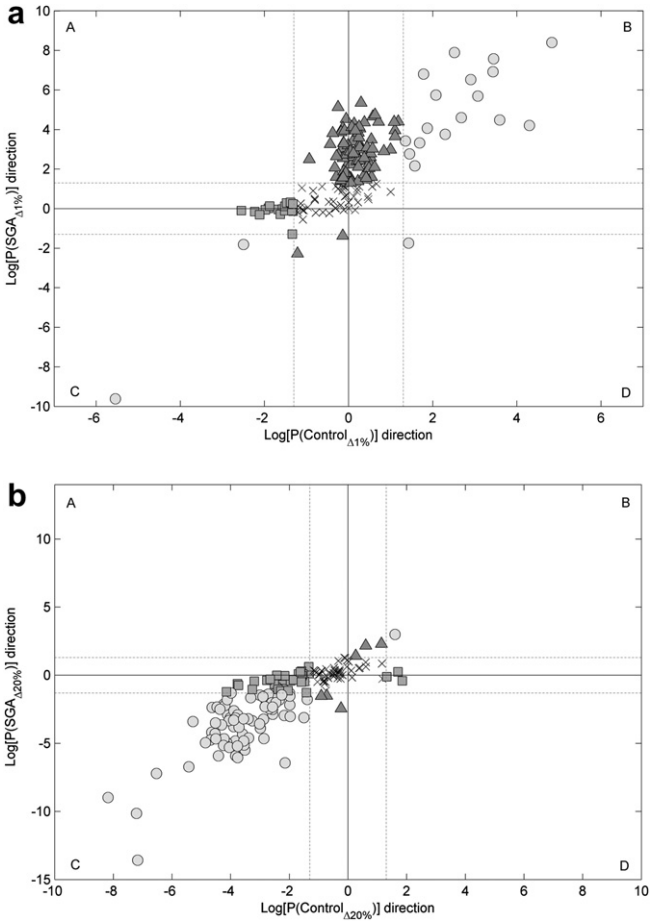

Fig. 3. (a) and (b) are biplots. Each point in the biplot represents one of the observed metabolite features. Each point's co-ordinate location (x, y), is defined by the significance of the relative change in metabolite concentration combined with the mean direction of that change (x = control; y = SGA). In these biplots, points lying in *zone A* show a mean increase in metabolite level for SGA samples and a mean decrease in control samples; *zone B* show a mean increase in metabolite level for both SGA and control samples; *zone C* show a decrease in mean metabolite level for both SGA and control samples; *zone D* show a decrease in mean metabolite level for SGA samples and an increase for control samples. (a) Biplot of change in metabolites, significant at 6% O_2, when explants were cultured at 1% O_2. The triangles (n = 81, 50%) show significant change in metabolite level for SGA samples but no significant change for control samples and the squares (n = 16, 10%) show significant change in metabolite level for control samples but no significant change for SGA samples (P = 0.05). (b) Biplot of change in metabolites, significant at 6% O_2, when explants were cultured at 20% O_2. The triangles (n = 6, 4%) represent significant change in metabolite level for SGA samples but no significant change for control samples and the squares (n = 31, 19%) represent significant change in metabolite level for control samples but no significant change for SGA samples (P = 0.05). A circle indicates a metabolite which significantly changes in both the SGA and control groups. The triangles indicate metabolites that are significantly changed in SGA but not significantly changed in control, and the squares indicate metabolites that are significantly changed in control but not significantly changed in SGA. The crosses indicate no significant change in either the SGA or control samples.

4. Discussion

4.1. The global differences in the metabolic footprint of the conditioned culture media were primarily dependent on the O_2 tension the samples were cultured under

The PC-CVA model built using all the detected metabolite features showed that the major differences in the metabolic footprint were due to the differing O_2 tensions (Fig. 1). This was to be expected, given the extreme differences in oxygen tensions in which the villous explants were cultured, and such metabolic changes have been demonstrated previously using this model [23,33] and tissue damage (including apoptosis) has been shown by exposing to hypoxic conditions [19].

4.2. The metabolic footprints of SGA and control conditioned culture media were different when compared at each O_2 tension in isolation

There was a significant difference between SGA and control classes when both were cultured at either 6% or 20% O_2 tensions. However, at 1% O_2 there was no significant difference between SGA and controls (Fig. 1). These multivariate findings were corroborated

Table 2
The table shows the 79 metabolites that showed a significant increase in SGA sample concentration but no significant change in the controls when culture conditions were changed from 6% to 1% O_2. The table shows the significance value of each metabolite at each oxygen tension and those with $p < 0.05$ are shaded. The *q*-value represents the False Discovery Rate for each metabolite. These 79 metabolites represent the triangles in quadrant A and B in Fig. 3(a). MG = monoacylglycerol; CE = cholesterol ester; PC = phosphocholine; PE = phosphoethanolamine; PI = phosphoinositol; PGE2 = prostaglandin E2; PGD2 = prostaglandin D2; TG = triglyceride/triacylglycerol. Those metabolites marked with an asterisk are definitely identified. Where PC or PE or PI is listed without a fatty acid configuration, it was not possible to give a more specific identification but they are classified as PC or PE or PI.

No.	*P*-Value 1%O_2	*q*-Value 1%O_2	*P*-Value 6%O_2	*q*-Value 6%O_2	*P*-Value 20%O_2	*q*-Value 20%O_2	Metabolites
1	0.0100	0.23	0.0004	0.02	0.0318	0.20	(homo)aconitate
2	0.0304	0.36	0.0012	0.03	0.0244	0.18	MG(16:0)
3	0.0388	0.39	0.0076	0.05	0.0566	0.25	UDP-2-acetamido-4-dehydro-2,6-dideoxyglucose OR Lithospermic acid
4	0.0142	0.27	0.0085	0.05	0.6847	0.67	2-Methoxyestrone 3-glucuronide OR 17-Propylestra-1,3,5(10)-triene-3, 17beta-diol diacetate OR Pregna-5,16,20-triene-3beta,20-diol diacetate
5	0.0153	0.28	0.0092	0.05	0.5914	0.63	2-Deoxystreptidine OR 14-Dihydroxycornestin OR Heptopyranosides; O-(2-acetamido-2-deoxy-alpha-D-galactopyranosyl)-L-serine OR 3-Hydroxydodecanedioic acid
6	0.0671	0.49	0.0001	0.01	0.0069	0.11	Hydroperoxy-11Z,13E-eicosadienoic acid OR 11-deoxy-PGF1a OR 11-deoxy-PGF1b OR PGF2α OR 1,2-heptadecanediol
7	0.8025	0.82	0.0009	0.03	0.0106	0.12	5alpha-Androstane-2beta-fluoro-17beta-ol-3-one acetate OR 6beta-Fluoro-5alpha-hydroxypregnane-3,20-dione OR 9-Fluoro-11beta,17beta-dihydroxy-2,17-dimethylandrost-4-en-3-one
8	0.7492	0.82	0.0014	0.03	0.0229	0.17	Tryptophan*
9	0.4357	0.82	0.0014	0.03	0.0073	0.11	Stearoylcarnitine OR Vitamin D derivative
10	0.8917	0.82	0.0019	0.03	0.0048	0.09	CE(20:4)
11	0.8862	0.82	0.0019	0.03	0.0209	0.16	*N*-(octadecanoyl)-tetradecasphing-4-enine-1-phosphoethanolamine OR CE(18:1) OR N-(tetradecanoyl)-1-β-glucosyl-sphing-4-enine
12	0.5575	0.82	0.0023	0.03	0.0014	0.07	PC or PE − more than 10 hits
13	0.0601	0.48	0.0024	0.03	0.0386	0.22	Oxoproline*
14	0.5885	0.82	0.0029	0.04	0.0211	0.16	3alpha,12alpha-Dihydroxy-5beta-chol-6-enoate
15	0.9284	0.82	0.0032	0.04	0.0359	0.20	PC(14:0/18:0) OR PC(O-16:0/16:0)
16	0.6607	0.82	0.0033	0.04	0.0116	0.13	PC or ubiquinone-8
17	0.7986	0.82	0.0052	0.04	0.0162	0.14	*N*-Glycoloylganglioside GM2 OR phosphocholine OR phosphoethanolamine
18	0.0870	0.56	0.0054	0.04	0.0312	0.20	(3s)-2,3,4,5-Tetrahydropyridin-3-amine OR Hypoxanthine
19	0.9258	0.82	0.0055	0.04	0.0074	0.11	1-O-α-D-glucopyranosyl-1,2-nonadecandiol OR 27-nor-5b-cholestane-3a,7a, 12a,24,25-pentol
20	0.8405	0.82	0.0056	0.04	0.0019	0.08	PC or PE − more than 10 hits
21	0.5472	0.82	0.0060	0.04	0.0104	0.12	1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine
22	0.8722	0.82	0.0073	0.05	0.0063	0.11	PC(16:0/dm18:0)
23	0.9021	0.82	0.0080	0.05	0.0009	0.07	PC or PE − more than 10 hits
24	0.6315	0.82	0.0080	0.05	0.0033	0.08	PC − more than 10 hits
25	0.8699	0.82	0.0085	0.05	0.0013	0.07	*N*-Glycoloylganglioside GM2 OR *N*-(2-hydroxyhexacosanoyl)-phytosphingosine OR PC
26	0.7200	0.82	0.0092	0.05	0.0418	0.23	Fucosamine OR D-quinovosamine OR Dideoxy-2,5-imino-D-glucitol OR Deoxy-D-glucosamine OR Dimethylaminopurine OR Hippurate OR *N*-Acetylanthranilate OR Trihydroxydihydroquinoline OR 3-succinoylpyridine OR adrenochrome OR 4-acetamidobenzoic acid
27	0.6983	0.82	0.0098	0.05	0.0037	0.09	PC − more than 10 hits
28	0.7382	0.82	0.0116	0.06	0.0329	0.20	PC(O-8:0/O-1:0)
29	0.7200	0.82	0.0128	0.06	0.0281	0.19	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-oxide
30	0.8346	0.82	0.0140	0.06	0.0432	0.23	Undecyl-phosphinic acid butyl ester OR Hexadecanal
31	0.7716	0.82	0.0143	0.06	0.0082	0.11	PC or PE − more than 10 hits
32	0.7154	0.82	0.0160	0.07	0.0307	0.19	Methionine*
33	0.7055	0.82	0.0166	0.07	0.0064	0.11	PI or PC − more than 10 hits
34	0.3970	0.82	0.0205	0.08	0.0003	0.04	PC or PE − more than 10 hits
35	0.7840	0.82	0.0308	0.09	0.0089	0.11	PC(18:3/18:1) OR PC(18:2/18:2) OR CE(22:5)
36	0.6375	0.82	0.0399	0.11	0.0125	0.13	PC − more than 10 hits
37	0.5698	0.82	0.0402	0.11	0.0132	0.13	PC − more than 10 hits
38	0.1398	0.70	0.0010	0.03	0.0669	0.27	beta-Alanine OR Sarcosine OR 2-Phosphoglycolate OR Hydroxy-threonine OR Hydroxymethylserine OR Oxoproline
39	0.7845	0.82	0.0012	0.03	0.0661	0.27	Octadecadienal OR Icosatetraenoic acid OR 3alpha-Hydroxy-2alpha-methyl-5alpha-androstan-17-one
40	0.7021	0.82	0.0018	0.03	0.2491	0.46	1α,25-dihydroxy-oxavitamin D3 OR 3alpha, 12alpha-Dihydroxy-5beta-cholan-24-oylglycine OR Chenodeoxyglycocholate OR Glycodeoxycholate; Glycochenodeoxy cholate
41	0.9714	0.82	0.0024	0.03	0.2173	0.44	dTDP-4-amino-4,6-dideoxy-D-monosaccharide
42	0.4364	0.82	0.0039	0.04	0.7876	0.70	*N*-Ethyl-5′-carboxamido adenosine
43	0.0775	0.52	0.0048	0.04	0.1634	0.39	Trihydroxybenzene
44	0.6748	0.82	0.0049	0.04	0.1248	0.35	PGE2 methyl ester OR PGD2-dihydroxypropanylamine
45	0.2889	0.82	0.0058	0.04	0.3285	0.50	Urate OR *N*-Propanoylimidazole OR Methylimidazole acetaldehyde
46	0.7206	0.82	0.0066	0.04	0.0675	0.27	Dibenzo[a,l]pyrene-11,12-diol-13,14-epoxide
47	0.6969	0.82	0.0082	0.05	0.2592	0.46	12-OPDA OR Oxo-phytodienoic acid OR 17beta-Hydroxy-2-oxa-5alpha-androstan-3-one

(*continued on next page*)

Table 2 (*continued*)

No.	*P*-Value 1%O_2	*q*-Value 1%O_2	*P*-Value 6%O_2	*q*-Value 6%O_2	*P*-Value 20%O_2	*q*-Value 20%O_2	Metabolites
48	0.7005	0.82	0.0107	0.05	0.8440	0.71	sn-glycero-3-Phosphoethanolamine OR (3-Arylcarbonyl)-alanine OR Phenylacetylglycine OR 5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-tetrahydroquinoline OR Methylhippuric acid
49	0.6825	0.82	0.0110	0.05	0.1135	0.33	1,3-Dimethyl-6,8-isoquinolinediol OR 3-Indolepropionic acid OR methyl indole-3-acetate
50	0.8872	0.82	0.0111	0.05	0.3555	0.52	PC – more than 15 hits
51	0.3280	0.82	0.0112	0.05	0.4763	0.58	1,3-Diaminopropane OR 2-Acetolactate OR Glutarate OR Deoxyribonolactone OR 4-Hydroxy-2-oxopentanoate OR 2-(Hydroxymethyl)-4-oxobutanoate OR 4,5-dihydroxy-2,3-pentanedione
52	0.6088	0.82	0.0147	0.06	0.0566	0.25	PC(O-14:0/O-16:0) OR PE-NMe(O-16:0/O-16:0) OR PC(15:0/18:1(11Z)) OR PE(14:0/22:1(13Z)) OR PE(14:1/22:0) OR PE(16:0/20:1) OR PE(16:1/20:0) OR PE(18:0/18:1)
53	0.7668	0.82	0.0189	0.07	0.0967	0.31	2-Naphthylamine-1-sulfonate OR 2-Phenylamino-ethanesulfonic acid
54	0.1597	0.71	0.0206	0.08	0.8008	0.70	3-Methylphenylalanine OR *N*,*N*-dimethyldopaminequinone
55	0.9165	0.82	0.0214	0.08	0.5682	0.62	*N*-Cyclohexyltaurine
56	0.9233	0.82	0.0231	0.08	0.2582	0.46	Hydroxysteroid OR 5alpha-Androstan-17beta-ol OR Isomer
57	0.9364	0.82	0.0241	0.08	0.0816	0.29	3-Methylcrotonylglycine OR Vinylacetylglycine OR *N*-Acetylproline OR Hippurate OR *N*-Acetylanthranilate OR Trihydroxy-5,6-dihydroquinoline OR 3-succinoylpyridine OR adrenochrome OR 4-acetamidobenzoic acid
58	0.4686	0.82	0.0258	0.09	0.1012	0.31	PC or PE – more than 10 hits
59	0.7030	0.82	0.0266	0.09	0.1338	0.35	Propylmalate; Dimethyl-hydroxyglutaric acid; Oxiranpseudoglucose; 5-Hydroxymethyl-chonduritol
60	0.8209	0.82	0.0274	0.09	0.2347	0.45	Tetracosahexaynoic acid OR 6-[5]-ladderane-1-hexanol
61	0.2376	0.82	0.0285	0.09	0.5741	0.62	Tricosanoic acid OR Methyl-docosanoic acid OR 3-Deoxyvitamin D3
62	0.6410	0.82	0.0297	0.09	0.2816	0.47	O-(4-Hydroxy-3,5-diidophenyl)-3,5-diiodo-tyrosine OR Thyroxine
63	0.0837	0.55	0.0299	0.09	0.7918	0.70	Heptadecanoyl carnitine OR Vitamin D3 derivative
64	0.8435	0.82	0.0300	0.09	0.2678	0.46	Hippurate OR *N*-Acetylanthranilate OR Trihydroxy-5,6-dihydroquinoline OR 3-Succinoylpyridine OR adrenochrome OR 4-Acetamidobenzoic acid
65	0.8948	0.82	0.0333	0.10	0.2427	0.46	Heptyl-beta-D-glucopyranoside
66	0.8485	0.82	0.0334	0.10	0.0821	0.29	PC or PE – more than 10 hits
67	0.8543	0.82	0.0347	0.10	0.1060	0.32	Undecaprenol
68	0.4124	0.82	0.0360	0.10	0.1704	0.39	2-Hydroxy-archaeol
69	0.9516	0.82	0.0375	0.10	0.1077	0.32	Phenylethylamine OR *N*,*N*-Dimethylaniline OR *N*-Ethylaniline
70	0.7615	0.82	0.0383	0.11	0.2792	0.47	1-Phosphatidyl-D-myo-inositol
71	0.8986	0.82	0.0394	0.11	0.6984	0.67	Phenylalanine
72	0.9001	0.82	0.0400	0.11	0.2558	0.46	epsilon-Caprolactam OR *N*-Formylpiperidine
73	0.6269	0.82	0.0402	0.11	0.0501	0.24	PC or PE – more than 10 hits
74	0.4044	0.82	0.0406	0.11	0.7473	0.68	5′-Dehydroadenosine OR 3′-Oxo-adenosine OR O-Succinylhomoserine OR *N*-Acetyl-D-mannosaminolactone
75	0.8799	0.82	0.0413	0.11	0.6464	0.65	C17 Sphinganine-1-phosphate OR Homophytanic acid OR Heneicosanoic acid OR Methyl-eicosanoic acid OR Dimethyl-nonadecanoic acid
76	0.8336	0.82	0.0453	0.11	0.0694	0.27	dTDP-D-mycaminose OR BIOTINOL-5-AMP;
77	0.9401	0.82	0.0458	0.12	0.1034	0.32	Tyrosine OR threo-3-Phenylserine OR 4-Hydroxy-4-(3-pyridyl)-butanoic acid
78	0.3392	0.82	0.0459	0.12	0.0529	0.25	*N*-Cyclohexyl-*N*′-Decylurea
79	0.7328	0.82	0.0490	0.12	0.0881	0.30	TG(16:1/16:1/17:2)

by the univariate statistical analysis. There were significant differences between the SGA and control classes at each individual O_2 tension, and a subset of metabolite features was significant across every oxygen tension (Fig. 2). However, this subset was relatively small (9% (52/574) of the total number of metabolite features that were significantly different at any O_2 tension).

4.3. Characterisation of the metabolic changes in the conditioned culture media suggests that changing from normoxic to hypoxic culturing conditions significantly increases disruption in SGA metabolism when compared with normal placenta

By performing pair-wise univariate analysis it was possible to characterise the nature of the changes in the medium metabolic signature due to decreasing the oxygen tension from 6% to 1%. Of the 161 metabolites that made up the normoxic biomarker signature of SGA, 49% exhibited a signature of SGA at 1% similar to that of the controls at 6% (Table 2). This suggests that, if placental hypoxia is the 'normal' *in vivo* condition for SGA as suggested by increased expression of hypoxia inducible factors, then exposure to normoxic oxygen levels significantly increases metabolic dysfunction in SGA placenta.

In addition, a small but significant number of metabolites (16; 10%) changed such that the control signature at 1% O_2 showed the same signature as SGA at 1% and 6%, but not the same as controls at 6% (where relative metabolite concentrations were significantly decreased) (Table 3). In this case, if placental hypoxia is the 'normal' *in vivo* conditions for SGA then, *for these metabolites only*, culturing control samples under hypoxic conditions induces the SGA metabolic signature. The metabolite numbers are small so this cannot be interpreted as a widespread trend.

4.4. Characterisation of the metabolic changes in the conditioned culture media suggests that changing from normoxic to hyperoxic culturing conditions has only a small effect on the metabolic differences between SGA and normal placenta

When looking at the changes in the medium metabolic signature due to increasing the O_2 tension from 6% to 20%, hyperoxia largely reduced the metabolite levels of both sample types while

Table 3
The table shows the 16 metabolites that showed a significant decrease in control sample concentration but no significant change in SGA when culture conditions were changed from 6% to 1% O_2. The table shows the significance value of each metabolite at each oxygen tension and those with $p < 0.05$ are shaded. The *q*-value represents the False Discovery Rate for each metabolite. These 16 metabolites represent the squares in Fig. 3(a). PC = phosphocholine; PE = phosphoethanolamine; CE = cholesterol ester; DG = diglycerides/diacylglycerol. Where PC or PE or PI is listed without a fatty acid configuration, it was not possible to give a more specific identification but they are classified as PC or PE or PI.

No.	*P*-Value 1%O2	*q*-Value 1%O2	*P*-Value 6%O2	*q*-Value 6%O2	*P*-Value 20%O2	*q*-Value 20%O2	Metabolites
1	0.0445	0.41	0.0153	0.06	0.2857	0.48	PC(14:0/2:0) OR PC(8:0/8:0) OR PE(9:0/10:0) OR LysoPC(20:5) OR Biliverdin
2	0.7452	0.82	0.0062	0.04	0.0350	0.20	3-O-(3,6-Anhydro-alpha-D-galactopyranosyl)-D-galactose 4-O-sulfate OR 4-O-beta-D-Glucosyl-4-hydroxycinnamate OR Coumarinic acid-beta-D-glucose
3	0.7690	0.82	0.0100	0.05	0.0440	0.23	Indole-3-acetate OR *N*-Acetylindoxyl OR 3-Indoleglycolaldehyde OR 5-Hydroxyindoleacetaldehyde
4	0.3755	0.82	0.0002	0.02	0.2796	0.47	3-Methylthiopropanal OR Tetrahydrothiophene 1-oxide
5	0.9779	0.82	0.0029	0.04	0.0769	0.29	Dolichyl diphosphate
6	0.9530	0.82	0.0033	0.04	0.9547	0.74	3-Deoxyvitamin D3
7	0.8752	0.82	0.0038	0.04	0.1426	0.37	*N*-(4-Guanidinobutyl)-4-hydroxycinnamide OR Dihydroconiferyl alcohol glucoside
8	0.1362	0.69	0.0038	0.04	0.1087	0.32	lysoPE(18:2) OR lysoPC(17:1)
9	0.9596	0.82	0.0062	0.04	0.8366	0.71	CE(18:2)
10	0.8974	0.82	0.0072	0.05	0.0671	0.27	9-Butyl-8-(3,4,5-trimethoxybenzyl)-9h-purin-6-amine
11	0.8902	0.82	0.0170	0.07	0.6221	0.64	4-(2-Amino-ethoxy)-2-[(3-hydroxy-2-methyl-5-phosphonooxymethyl-pyridin-4-ylmethyl)-amino]-but-3-enoic Acid; 6-Hydroxy-5-methoxyindole glucuronide OR 5-Hydroxy-6-methoxyindole glucuronide OR 4-Hydroxy-6-methylpretetramide OR Alanyl-poly(glycerolphosphate)
12	0.8971	0.82	0.0203	0.08	0.2622	0.46	Tefluthrin
13	0.9458	0.82	0.0223	0.08	0.0583	0.25	DG − more than 10 hits
14	0.7392	0.82	0.0283	0.09	0.4184	0.56	CE(18:0) OR CE(20:3) OR CE(22:6)
15	0.6602	0.82	0.0331	0.10	0.7649	0.69	15:0 Cholesteryl ester
16	0.5223	0.82	0.0332	0.10	0.6401	0.65	Creatine OR 3-Guanidinopropanoate OR O-ureidohomoserine

keeping the metabolic signature difference between SGA and control intact (48%), suggesting a general increase in catabolism of nutrients under 20% O_2 tension conditions. Overall, three quarters (75%) of metabolites showed no changes or concordant change implying that the SGA samples do not differentially respond to this increase in oxygen tension and that control samples differentially change only minimally.

The metabolites that have been identified (Table 2 and Supplementary file) may provide further understanding of the pathophysiology of SGA. Although due to the novelty of these studies, the significance of many of these metabolites in relation to SGA is uncertain as they have not been detected previously. A wide range of metabolism is disrupted including a number of metabolites (16 identified putatively) at all oxygen tensions. The most interesting results were found when culture conditions were changed from 6% to 1% O_2 tension as described in Section 4.3 (see Tables 2 and 3). An in depth discussion of all these metabolites is not feasible in this article but some metabolites will be considered further here.

A metabolite class that showed much disruption (upregulation) in the culture medium of SGA samples when conditions were changed from 6% to 1% was the phospholipids. Phospholipids are the major lipid constituents of cell membranes. Glycerophosphocholines and glycerophosphoethanolamines are two of the common phospholipids as has been observed for the placenta [40]. Changes in normal oxygen tensions can cause changes to glycerophospholipids resulting in many different products which have many different proposed biological properties [41]. Phosphatidylcholine metabolism is affected by hypoxia particularly in the heart in animal studies [42] but there is a lack of evidence on the effect of hypoxia on glycerophospholipids in the placenta. The phospholipid changes observed in the described study are most likely a result of cell membrane damage leading to the subsequent release of phospholipids. However, there is recent evidence of anti-phospholipid antibodies (and complement activation) co-operating in triggering a local inflammatory process, eventually leading to placental thrombosis, hypoxia, and neutrophil infiltration [43].

A number of essential amino acids were also increased in SGA samples when culture conditions were changed from 6% to 1% including tryptophan, methionine and phenylalanine. Tryptophan functions as a biochemical precursor for serotonin and niacin. Of particular interest to SGA is that tryptophan is a precursor of serotonin. Elevated tryptophan and serotonin metabolism has been reported in plasma from newborn SGA infants [44] and in the brain of malnourished rat fetuses [45] and serotonin and its receptors have been localized in the placenta implying a role in placental development [46]. Kynurenine is a metabolite of tryptophan and kynurenine metabolism is significantly altered in fetal sheep when placental function is chronically compromised in late gestation [47]. Kynurenine was noted to be significantly decreased in SGA at these culture conditions but was also decreased in controls. This may be explained by indoleamine 2,3-dioxygenase activity which is involved in kynurenine metabolism. This has been localized in the placenta and detected from 14 weeks gestation and its placental activity was found to be lower in FGR [48]. Kynurenine was also noted to change by Dunn et al. in their study investigating metabolic disturbances in placental explants in pregnancies complicated by pre-eclampsia and in response to hypoxia [33].

Methionine is an essential amino acid required for normal growth and is a precursor of homocysteine. Hyperhomocysteinemia in particular is associated with a number of complications including SGA and cardiovascular disease and can result from a variety of acquired factors (deficiency of vitamins B6, B12 and folic acid, high meat diet, smoking and others) or genetic factors (abnormalities of methionine − homocysteine metabolism) [49]. The exact mechanism by which homocysteine is associated with vasculopathy is still unclear. Accumulating evidence suggests that hyperhomocysteinaemia alters cellular structure and metabolism, mediated by free radicals generated during the oxidation of homocysteine [50]. *In vitro* studies show that exposure of endothelial cells to homocysteine results in oxidative effects, including generation of superoxide anion radicals and

hydrogen peroxide, which lead to inactivation of nitric oxide-mediated pathways and endothelial-cell damage [51].

Phenylalanine is an essential amino acid and the precursor for the amino acid tyrosine. Phenylalanine has been found to be lower in umbilical venous samples from growth restricted pregnancies [52] and also in umbilical venous samples from growth restricted twins where there was discordant growth among monochorionic twins [53]. Another study showed significantly higher concentrations of phenylalanine ($P < 0.05$), in the SGA compared to the term normotensive and pre-eclamptic groups in placental villi homogenates [54]. Two of these studies used high performance liquid chromatography for the analysis [53,54]. These support our findings and suggest that there are abnormalities in placental metabolism, amino acid transfer into the fetal circulation, and also fetoplacental perfusion in pregnancies complicated by SGA. Changing O_2 tension has an effect on this placental metabolism but it is uncertain as to the extent of this effect.

Interestingly, of the metabolites that changed in the control samples when culture conditions were changed from 6% to 1% a number of cholesterol esters were identified. Cholesterol esters are cholesterol molecules with long-chain fatty acids linked to the hydroxyl group. Cholesterol esters are major constituents of the lipoprotein particles carried in blood. They also accumulate in the fatty lesions of atherosclerotic plaques. Low cholesterol ester concentrations and low high density lipoprotein levels have been found in SGA newborns and in the umbilical venous blood of SGA babies [55,56]. Cholesterol ester transfer has also been found to be increased in SGA neonates [57]. This increased cholesteryl ester transfer may in part, explain the increased risk of coronary heart disease (CHD) of small for gestational age neonates in later life.

We acknowledge several limitations of our study. SGA has been defined here as birth weight less than the 10th centile. A proportion of SGA infants defined as less than the 10th centile will be constitutionally small and thus there are limitations in the use of the birth weight percentile as a surrogate marker of FGR. However, in this study we used individualised centiles and all the cases had an IBR $\leq$the 5th centile, significantly increasing the probability that these cases resulted from placental dysfunction rather than being constitutionally small.

A significant challenge of metabolomic studies is metabolite identification. UPLC–MS is a very sensitive analytical technique. Further studies will be performed to establish the identity of features with multiple possible identifications.

This study has used metabolomic technologies to show differences between SGA and normal pregnancies under different oxygen conditions. To our knowledge this is the first time that metabolomics has been used to investigate SGA. The results from *in vitro* culturing of placental villous explants suggest that placental hypoxia is the likely *in vivo* condition for SGA but that simply restoring 'normoxia' may be deleterious to the tissue. Oxygen delivery to the placenta of an SGA fetus is likely to be a changing variable and the placenta of an SGA fetus is possibly more tolerant to low oxygen tensions. This may be similar to placental adaptation to chronic hypoxia at high altitude [32]. There are also a number of metabolites that are significantly different between SGA and controls at all oxygen levels, suggesting that other factors such as nutrient deficiency may play an important role in the pathophysiology of SGA. This may provide a clear differentiation between SGA and PE, where hypoxia was observed to be the dominant determinant of the phenotype [33]. There is also some overlap in disrupted metabolic classes between our study and the study by Dunn et al. [33]. It requires further clarification whether these changes are the result of a general disruption in SGA or PE-complicated pregnancies or are specific to SGA. We also wish to see how our findings relate to early pregnancy in women who go on to deliver an SGA baby.

This study further adds to the growing data from pregnancy metabolomics research. In summary, we have shown that the metabolic footprint of placental tissue exhibits different phenotypes when cultured at different O_2 tensions and moreover the metabolic profile of SGA placental explants can be distinguished from controls irrespective of O_2 tension. These results may provide some insight into the pathophysiology of this complex condition and will provide direction for future research to aid the pathophysiological understanding of SGA.

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Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.placenta.2010.07.002.

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