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Analysis of the Metabolic Footprint and Tissue Metabolome of Placental Villous Explants Cultured at Different Oxygen Tensions Reveals Novel Redox Biomarkers

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Abstract

Pre-eclampsia (PE) is a multi-system disorder of pregnancy hypothesised to arise from circulating factors derived from an unhealthy placenta. Some changes in placental phenotype seen in PE can be reproduced by culture in altered oxygen (O_2) tension. Currently, these circulating factors are unidentified, partly due to the complexity of maternal plasma. Investigation of factors released from placental tissue provides a potential method to identify bioactive compounds.

Experimental strategies to study compounds present in a biological system have expanded greatly in recent years. Metabolomics can detect and identify endogenous and secreted metabolites. We aimed to determine whether metabolites could be identified in placental cultures with acceptable experimental variability and to determine whether altered O_2 tension affects the composition of the placental metabolome.

In this study we used gas-chromatography-mass spectroscopy to determine the presence of metabolites in conditioned culture medium (CCM) and tissue lysates of placental villous explants cultured in 1, 6 and 20% atmospheric O_2 for 96 h. This experimental strategy had an intra-assay variation of 6.1–11.6%. Intra and inter-placental variability were 15.7–35.8% and 44.8–46.2% respectively. Metabolic differences were identified between samples cultured in 1, 6 and 20% O_2 in both CCM and tissue lysate. Differentially expressed metabolites included: 2-deoxyribose, threitol or erythritol and hexadecanoic acid. We conclude that metabolomic strategies offer a novel approach to investigate placental function. When conducted under carefully controlled conditions, with appropriate statistical analysis, metabolic differences can be identified in placental explants in response to altered O_2 tension. Metabolomics could be used to identify changes in conditions associated with placental pathology.

Keywords: Placental villous explants; Oxygen; Metabolomics; 2-Deoxyribose; Metabolic footprinting

1. Introduction

Pre-eclampsia (PE) affects 3–5% of pregnancies in the developed world and increases fetal and maternal morbidity and mortality. PE is a multi-system disorder, characterised by maternal systemic endothelial dysfunction and an exaggerated inflammatory response [1] and is thought to result from circulating factors originating from the placenta [2]. This hypothesis has developed because the presence of a placenta (but not a fetus) is required to develop PE [3], and the syndrome resolves after delivery or resorption of placental tissue [4].

PE is associated with changes in placental villi, particularly the syncytiotrophoblast, including: increased apoptosis [5],

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a loss of syncytiotrophoblast area [6] and increased density of syncytial knots [7]. There is also an increase in circulating syncytiotrophoblast debris, which may elicit a maternal immune response [8]. The most likely source of this placental damage is altered oxygen (O_2) delivery to the placenta, resulting in chronic low oxygenation or generation of reactive oxygen species [9]. This hypothesis has arisen from evidence demonstrating shallow invasion and limited remodelling of maternal uteroplacental spiral arteries [10] culminating in reduced placental blood flow. Some aspects of the placental phenotype, including the generation of circulating factors [11] can be reproduced *in vitro* by culture in low atmospheric O_2 (1–3%), in response to hypoxia-reoxygenation or following treatment with ROS [12–15]. Therefore, analysis of secreted factors from cultured placental tissue subject to altered oxygenation may provide a useful method to identify molecules of placental origin, which may contribute to the pathogenesis of PE.

Strategies to detect and identify compounds present in an experimental cellular system or organism (including transcriptomics, proteomics and metabolomics) have expanded considerably in recent years. The objective of metabolomics is the detection and identification of endogenous and exogenous metabolites to define the genotype or phenotype of a biological system [16–20]. The metabolome is the complete quantitative collection of small molecular weight compounds, which participate in metabolism or absorbed from the external environment [21,22]. Investigation of the metabolome is advantageous as it represents the final downstream product of cell function and as such may be a closer reflection of phenotype than the genome, transcriptome or proteome [17,20,23]. For example, small or undetectable changes in enzyme concentration could have a sizeable impact upon the concentration (but not necessarily the flux) of metabolic intermediates, as described from first principles by metabolic control analysis (MCA) [24,25]. Finally, the metabolome represents a smaller and more tractable group of compounds compared to the proteome or genome. Current estimates indicate the human metabolome consists of 1500–3000 endogenous metabolites (not including lipids and exogenous metabolites) [26,27] in contrast to 20,000–25,000 genes.

Currently, metabolomics is a hypothesis generation strategy, as there is limited knowledge of expected metabolic differences [28]. Typically, metabolic profiling is used to detect a wide range of metabolites covering a number of different metabolic classes to provide as large an overview of metabolism as achievable. After a well-designed experiment is performed, the data is interrogated to define metabolic differences observed [29,30]. The intracellular metabolome (endometabolome) and extracellular metabolome (exometabolome or 'metabolic footprint' [31,32]) can be studied, giving clues to metabolic pathways utilised within the cell and the effects cells may be having on their environment through released products [31,33–35].

The metabolome is a large collection of metabolites with a wide range of structures, chemical properties (volatility, polarity and solubility) and concentrations. This complexity necessitates the use of analytical tools and methodologies of high sensitivity, high separation efficiency (specificity) and with the ability to detect metabolites over a wide range of concentrations (typically, mM to sub-μM). Optimal analysis of the metabolome is most often achieved using chromatography to separate metabolites according to their physico-chemical properties coupled to mass-spectrometric (MS) detection [23]. Gas-chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) are commonly utilised to separate metabolites. GC-MS is commonly employed in metabolic profiling [23] and has been used to identify differences between maternal plasma from normal pregnancies and those complicated by PE [36].

We propose that metabolomics may provide a means to identify differences in the metabolic footprints of placental explants exposed to different culture conditions. To assess the feasibility of this technique we cultured placental villous explants in different O_2 tensions and investigated the metabolic footprint in conditioned culture media and the intracellular metabolome of placental tissue. The specific aims of these investigations were: (1) to determine whether metabolites could be reproducibly detected in samples of placental origin; (2) to determine the technical and biological variability of this approach; and (3) to determine whether altered O_2 tension affects the metabolic footprint and intracellular metabolome.

2. Methods

2.1. Culture of placental villous explants and collection of tissue and conditioned culture medium

Unless otherwise stated reagents were obtained from Sigma-Aldrich Chemical Co, Poole, UK. Following approval from the local research ethics committee, placentas were obtained from uncomplicated term pregnancies with the donors' written consent ($n = 11$). The placentas were collected within 20 min of delivery. Three areas of each placenta were sampled randomly using a sampling frame with intersecting lines at 10 cm, which was placed on the maternal surface of the placenta and placental tissue taken from underneath each intersection. The decidual surface and chorionic plate was removed, and the villous tissue was dissected into explants weighing approximately 10 mg per fragment. Three explants, one from each area sampled, were suspended on a single Netwell insert (Corning Inc, NY, USA) in CMRL-1066 culture medium supplemented with antibiotics, insulin (1 mg/l), hydrocortisone (0.1 mg/l), retinol acetate (0.1 mg/l) and 10% fetal calf serum. To obtain sufficient material for study 9-wells were used for each experiment. The explants were suspended at the gas-liquid interface as previously described [15]. Explants were cultured for a total of 96 h in 1, 6 or 20% O_2. After 48 h the culture medium was replaced with fresh medium already equilibrated for 24 h under the appropriate O_2 conditions. Explants cultured in 1% O_2 remained within this environment when cultured medium was changed. After 96 h, the conditioned culture medium was collected, immediately frozen, and stored at −80 °C. The explants were weighed, snap frozen, and then stored at −80 °C. The experiments were conducted in two batches (batches 1 and 2), each using a different batch of culture medium, prepared with different fetal calf serum.

2.2. Extraction of placental explants

Placental tissue was defrosted on ice, then homogenised with a pestle and mortar in ice-cold 50% methanol (v/v) in phosphate buffered saline for 2 min. The suspension was centrifuged at 9500 × *g* for 3 min at 4 °C, the supernatant

was transferred to a separate Eppendorf tube, and the process repeated. Finally, the two supernatants were combined.

2.3. Preparation of conditioned culture medium and placental explant extractions for GC-MS

To allow normalisation of response variability, conditioned culture medium was prepared for gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analysis by spiking 200 μl aliquots of cell-free supernatant with 100 μl internal standard solution (0.17 mg/ml succinic d_4 acid). Intracellular extracts from placental tissue were prepared by spiking 700 μl with 100 μl internal standard solution (0.16 mg/ml succinic d_4 acid). Both preparations were lyophilised using a HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap (Thermo Life Sciences, Basingstoke, UK). Immediately prior to GC-TOF-MS analysis, samples were chemically derivatised in a two-stage procedure. *O*-methylhydroxylamine solution (50 μl, 20 mg/ml in pyridine) was added and heated at 40 °C for 90 min followed by addition of 50 μl MSTFA (*N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide) with heating at 40 °C for 90 min. To the derivatised solution 20 μl of a retention index (RI) solution (0.6 mg/ml C_{10}, C_{12}, C_{15}, C_{19} and C_{22} *n*-alkanes) was added to allow normalisation of retention times.

2.4. GC-TOF-MS analysis

All samples were analysed using an Agilent 6890 gas chromatograph and 7673 autosampler (Agilent Technologies, Stockport, UK), interfaced to a LECO Pegasus III mass spectrometer (LECO Corp., Stockport, UK). All samples were analysed in a random order within 24 h of derivatisation using a previously described methodology employed for analysis of yeast metabolic footprints [37].

2.5. Data preprocessing and data analysis

Two samples from each class and each sample set (18 samples in total) were chosen and chromatographic peak deconvolution performed (LECO ChromaTOF v2.25 software) using the following parameters: peak width 3 s, baseline = 1, smoothing = 3. Each chromatographic peak defined by an Analyst (WBD) was added to a database with associated retention index and mass spectrum. In total this list amounted to 264 metabolite peaks. Subsequently, chromatographic peak deconvolution was performed for each sample and peaks present in the database were matched to those in the samples within certain criteria (RI ±10, mass spectral match >700). For those peaks detected, the peak area was calculated and normalised using the internal standards generating a response ratio (peak area-metabolite/peak area-internal standard). All data were exported as ASCII files to Microsoft Excel for data analysis.

As previously described [33], chemical identification of detected metabolite peaks was performed by searching mass spectral libraries (NIST/EPA/NIH02 http://www.nist.gov/srd/nist1a.htm), the Golm metabolite library (http://csbdb.mpimpgolm.mpg.de/csbdb/gmd/msri/gmd_contributions.-html) and a mass spectral/RI library (prepared by WBD) containing over 500 entries. Identifications were labelled as preliminary for spectra matched in the NIST/EPA/NIH02 or Golm metabolite libraries with a match score greater than 700. Identifications were labelled as definitive if the mass spectrum and retention index matched those of a metabolite present in WBD's library, which was prepared by analysis of authentic chemical standards (purchased from Sigma-Aldrich or Acros Chemicals, UK) on the instrument employed in this study.

2.6. Statistical analysis

Multivariate analysis was performed to determine the origin of variation between samples. This was then extended by the use of univariate analysis to determine whether the concentrations of individual metabolites differed for the metabolic footprint or intracellular metabolome, under different O_2 tensions.

Within Matlab® (http://www.mathworks.com), exploratory multivariate analysis was performed using principal components analysis (PCA), an unsupervised approach which transforms a large set of related variables into a new, smaller set of independent variables, termed principal components (PCs). Each PC represents an axis in multidimensional space and corresponds to the direction of maximum variation of the original data. PCA was performed on data normalised to zero mean and unit variance, so that results were not dominated by a small number of high intensity peaks but gave equal weighting to peaks of low intensity.

Univariate statistical analysis was performed, using the non-parametric Kruskal–Wallis, Friedman test and Mann–Whitney *U*-test to calculate whether there were statistically significant differences for any given peak between samples cultured under different O_2 tensions. The critical *p*-value for rejecting the null hypothesis in a single test is usually 0.05. However, where many metabolites are tested in parallel, the *p*-value for rejecting the individual hypothesis is typically reduced to lower the probability of type 1 errors (false positives). Therefore, a *p* value of 0.01 was used in these experiments.

3. Results

3.1. Variability of experimental techniques

To assess experimental variability, GC-TOF-MS analysis was performed on one sample set, termed batch 1, constructed from five placentas for each O_2 tension. The median intra-assay variability was calculated from the triplicate analysis of one sample for each O_2 tension (1, 6 and 20%). The range of median intra-assay coefficients of variation (COV, $n = 3$) for batch 1 was 6.1–11.6%. The median intra-placental variability, the biological difference between samples from the same placenta cultured under the same conditions was in the range 15.7–35.8% ($n = 3$). The median inter-placental variability, the difference between samples from different placentas cultured under the same O_2 tension, was in the range 44.8–46.2% ($n = 5$). The interclass variability for all placentas cultured under all O_2 tensions was 53.1% ($n = 15$).

GC-TOF-MS analysis was subsequently performed on a second sample set, termed batch 2. Four samples from batch 1 were reanalysed at the same time as batch 2 to reduce technical variability. To identify the source of greatest variation within the combined data for batches 1 and 2, multivariate analysis was performed. PCA showed separation of samples according to the batch in PC1, i.e. the four samples in batch 1 differ from the six samples in batch 2 (Fig. 1). This difference accounts for 32.3% of variance within the combined sample set and is the highest single source of variability in this combined set. Therefore, 66.7% of the variance is attributable to other factors (Figs. 1 and 2). Also, conditioned culture medium sampled at 96 h from batch 1 differed from medium sampled at 0 h (data not shown), indicating that culture produced detectable biological changes in the metabolic footprint.

3.2. Effect of O_2 tension on the metabolic footprint (exometabolome)

The metabolic footprint in conditioned culture medium from placentas were analysed in two sample sets containing five and six samples of conditioned culture medium, respectively in batches 1 and 2. Analysis detected 264 unique metabolite peaks. Univariate analysis was used to compare metabolite concentrations in samples cultured in different O_2 tensions for batch 1. Five metabolites were identified as significantly

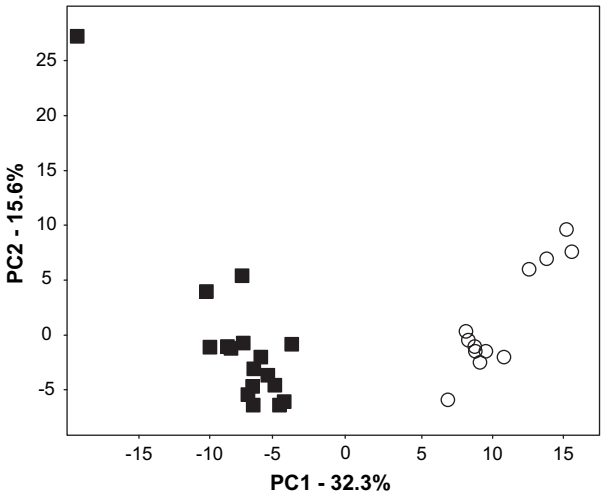

Fig. 1. Principal components analysis demonstrating separation between metabolic footprints of conditioned culture medium from two different experiments (March ○ and June ■) analysed at the same time. Data for four placental samples each cultured at 1, 6 and 20% O_2 tensions were re-analysed for batch 1 and six placental samples each cultured at 1, 6 and 20% O_2 tensions for batch 2. One replicate for 20% O_2 tension for batch 2 was removed as an outlier.

different between samples cultured at 1 and 6% O_2 (Table 1) and this number increased to 15 between the larger difference of 1 and 20% O_2 (Table 2). No metabolites were found to be significantly different between explants cultured in 6 and 20% O_2. Where possible, significant peaks were chemically identified.

Table 1
Metabolite peaks observed to be statistically different when comparing the metabolic footprints detected for O_2 tensions of 1 and 6% in two different batch experiments

Metabolite	Batch 1	Batch 2	Discriminatory peak in both batches
	p-value	*p*-value	
Threonic acid (65)	**0.009023**	0.423340	–
2-Deoxyribose (peak 1 69)	**0.009023**	**0.006485**	**Yes**
2-Deoxyribose (peak 2 70)	**0.009023**	**0.006485**	**Yes**
Peak 80 unidentified	0.916815	**0.003948**	–
Peak 111 unidentified	**0.009023**	0.386476	–
Peak 140 unidentified	**0.009023**	0.016309	–
Hexadecanoic acid (147)	**0.009023**	**0.003948**	**Yes**

The metabolites highlighted in bold are statistically significant.

Three metabolites were significantly elevated in samples from 1% O_2 ($p < 0.01$) compared to 6% O_2: 2-deoxyribose, threonic acid and hexadecanoic acid.

Batch 2 was used as a validation sample set to confirm or refute the results observed from batch 1. Again, univariate analysis was used to compare metabolite concentrations in samples cultured in different O_2 environments, with significant peaks being chemically identified where possible. Three metabolites were identified as significantly increased between samples cultured at 1 and 6% O_2 (Table 1) and this number increased to 12 between the larger difference of 1 and 20% O_2 (Table 2). No metabolites were found to be significantly different between explants cultured in 6 and 20% O_2. A number of peaks were highlighted as different in both batches 1 and 2.

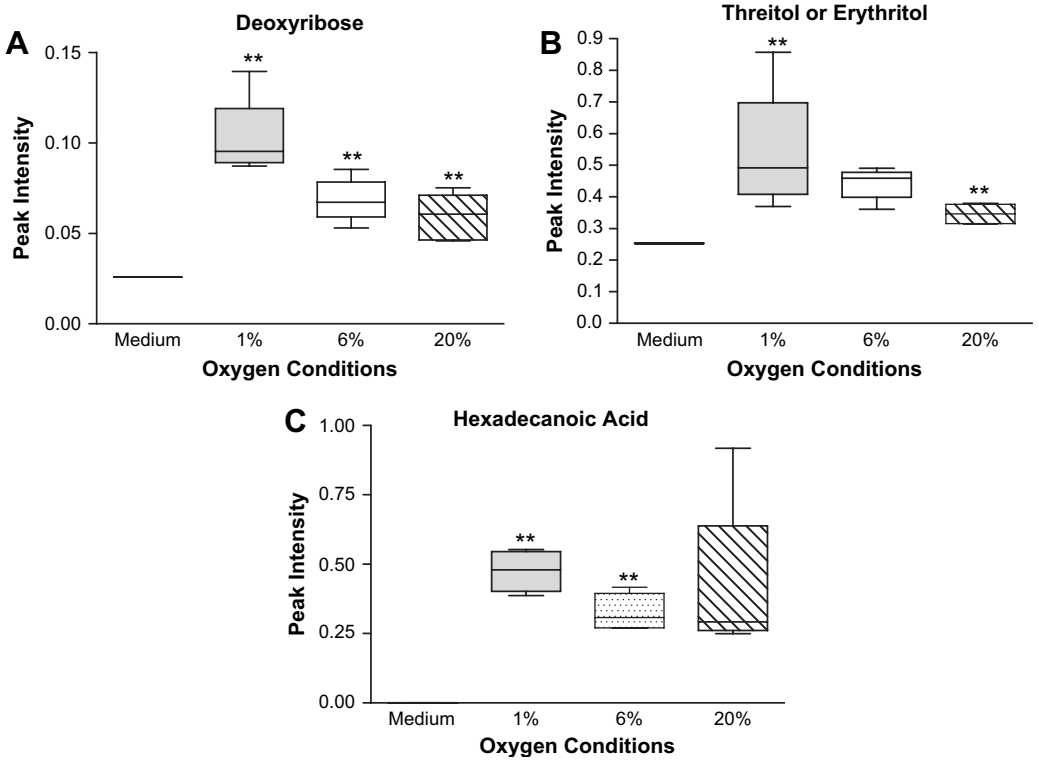

Fig. 2. Box and whisker plots demonstrating altered expression of metabolites in conditioned cultured medium in batch 1 in response to different atmospheric O_2 tensions. (A) Deoxyribose, (B) threitol or erythritol, (C) hexadecanoic acid, $^{**}p < 0.01$ Kruskal–Wallis test.

Table 2
Metabolite peaks observed to be statistically different when comparing the metabolic footprints detected for O_2 tensions of 1 and 20% in two different batch experiments

Metabolite	Batch 1	Batch 2	Discriminatory peak in both batches
	p-value	*p*-value	
Ethanolamine (17)	0.347208	**0.006170**	–
Glycerol (19)	**0.009023**	0.201243	–
Phosphate (37)	**0.009023**	0.075800	–
Threitol/erythritol (51)	**0.009023**	**0.006170**	**Yes**
Peak 52 unidentified	**0.009023**	0.067889	–
Gluconic acid (59)	**0.009023**	0.044610	–
Threonic acid (63)	**0.009023**	0.327187	–
Threonic acid (65)	**0.009023**	0.361310	–
2-Deoxyribose (69)	**0.009023**	**0.006170**	**Yes**
2-Deoxyribose (70)	**0.009023**	**0.006170**	**Yes**
Peak 77 unidentified	0.754023	**0.006170**	–
Peak 80 unidentified	0.347208	**0.006170**	–
Peak 91	**0.009023**	0.100348	–
Peak 93	**0.009023**	ND	–
Peak 96	**0.009023**	ND	–
Peak 111	**0.009023**	0.248213	–
Peak 114 (sugar)	**0.009023**	**0.006170**	**Yes**
Peak 115	0.288844	**0.006170**	–
Peak 116	**0.009023**	0.301700	–
Quinic acid (136)	0.025347	**0.006170**	–
Peak 140	0.016294	**0.006170**	–
Hexadecanoic acid (147)	**0.009023**	0.100348	–
Octadecanoic acid (154)	**0.009023**	0.144127	–
Peak 225	ND	**0.006170**	–
Peak 230	ND	**0.006170**	–
Peak 284	ND	**0.006170**	–

The metabolites highlighted in bold are statistically significant.

For samples cultured at 1 and 6% O_2, 2-deoxyribose and hexadecanoic acid were significantly increased in both batches. For samples cultured at 1 and 20% O_2, threitol or erythritol, 2-deoxyribose, an unidentified sugar and one unidentified peak were significantly increased in both batches (Tables 1 and 2, Figs 2A–C).

3.3. Effect of O_2 tension on the intracellular metabolome (endometabolome)

Explant tissue homogenate from batch 2 ($n = 6$) was analysed in a single GC-MS run detecting 127 metabolites. Univariate analysis (Friedman and Kruskal–Wallis) identified metabolites that were present in significantly different concentrations in explants cultured at different O_2 tensions. Five metabolites were significantly different between 1, 6 and 20% O_2 tension using the Friedman test (Table 3). In explants 2-deoxyribose and threitol/erythritol were significantly different in altered O_2 (Fig. 3), as well as three unidentified metabolites. For these experiments, the concentration of hexadecanoic acid was regarded as non-significantly elevated in 1% compared to 6% O_2 ($p = 0.016$).

4. Discussion

Metabolomics has expanded rapidly into new scientific fields in recent years. Despite this expansion, there is only one report of metabolomics investigations of placental tissue [38] and there are no reports of metabolomic (metabolic footprint) investigations of placental tissue following exposure to altered oxygenation. In these preliminary studies we aimed to determine whether metabolomic strategies were appropriate for the investigation of placental tissue, including the comparison of technical and biological variability. We utilized a tissue culture model, which reproduces several aspects of the abnormal cell turnover observed in pre-eclampsia and alters the release of proteins such as hCG and sFlt-1 [11,12,15]. Metabolomics identified differences between fresh culture medium and conditioned medium from placental explants and also identified differences between metabolic footprints obtained at different O_2 tensions. This indicates sufficient sensitivity in detecting changes in placental tissue culture.

Our first aim was to determine whether we could detect metabolites in the placental metabolic footprint. A total of 264 unique metabolites were detected in batches 1 and 2, of which 101 were chemically identified by matching retention indices and electron impact mass spectra to those of authentic standards. The median intra-assay COV was similar to other metabolic profiling studies [39]. The interclass COV (53.1%) was greater than the intra-placental COV (15.7–35.8%) and within-class inter-placental COV (44.8–46.2%). This demonstrates that variability associated with technical processes (sample preparation and instrumental analysis) is lower than variability associated with differences between biological samples. This is a requirement of metabolomic investigations. In addition, intra-placental variation was less than inter-placental variation showing that collection of data from different placentas is required, rather than collection of multiple data from the same placenta. Within-class inter-placental variation was less than between-class inter-placental variation showing that there are greater differences between classes (between placenta cultured at different O_2 tensions) than within classes (placenta cultured at the same O_2 tension), necessary to deduce metabolic changes between the experimental classes. These results show that metabolomic methodologies are suitable for distinguishing metabolic variations in culture of explants at different O_2 tensions. They also indicate that when appropriate experimental design is applied, observed differences are likely to originate from true biological variation rather than technical inaccuracies.

The placental collection occurred over a period of several months and the culturing of placental tissue was required immediately (without storage). To this end we performed two separate experiments of sample sizes of $n = 5$ (batch 1) and $n = 6$ (batch 2) rather than a combined analysis of $n = 11$. These batches were collected and cultured 3 months apart by two separate researchers (AH-batch 1 and SW-batch 2). When these batches were analysed at the same time, PCA of the combined dataset showed separation of batches 1 and 2 in PC1. The most likely explanation is the use of different batches of growth medium; this has been observed for different batches of media in other studies by the authors (unpublished data, WBD). We hypothesise that this variation arose from different batches of fetal calf serum and propose the

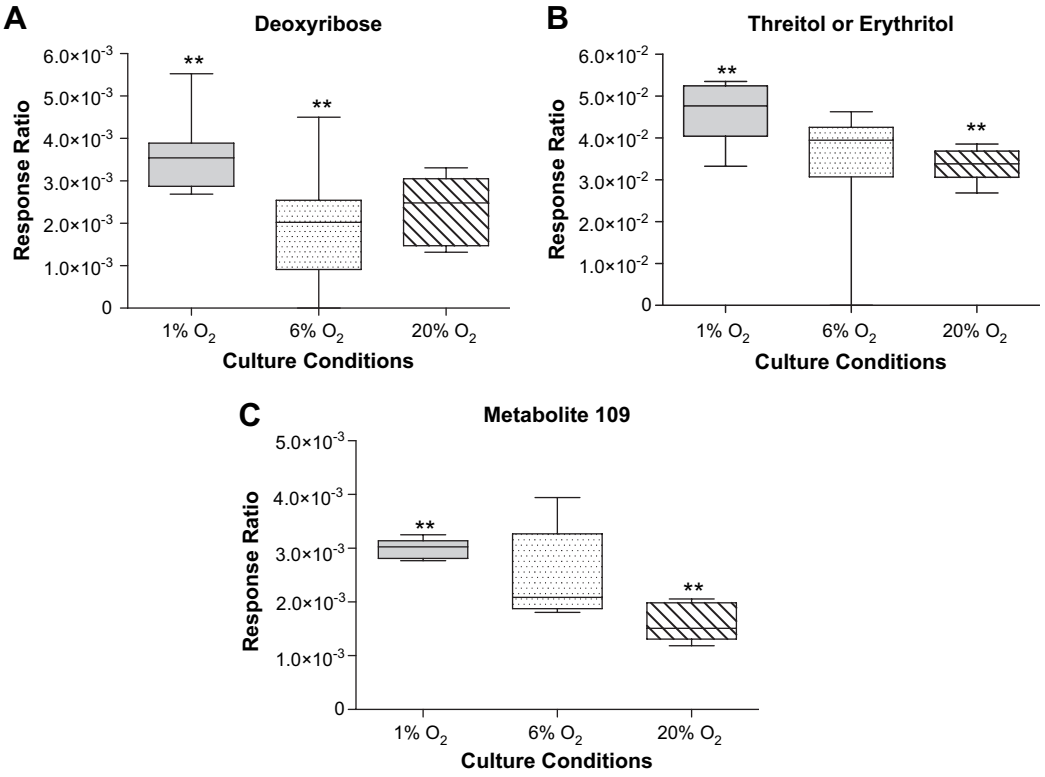

Fig. 3. Box and whisker plots demonstrating altered expression of metabolites in placental lysate in batch 2 in response to different atmospheric O_2 tensions. (A) Deoxyribose, (B) threitol or erythritol and (C) metabolite 109 (**$p < 0.01$).

use of identical batches of growth medium for all future experiments.

The final aim of these experiments was to determine whether GC-MS could define differences in the endo- and exometabolome of placental villous explants exposed to different atmospheric O_2 tensions. Using an appropriate level of statistical significance ($p < 0.01$) no differences were demonstrated in the endo or exometabolome between villous explants cultured in 6 and 20% O_2. This suggests that culture in O_2 levels exceeding those estimated as physiological for term human placenta (6–8%) may not significantly alter metabolic function [40].

In contrast, when each batch was analysed separately several metabolites were significantly elevated in samples conditioned at 1% O_2 compared to those at 6 and 20% O_2. In these experiments, batch 2 was used as an independent sample set to validate results from batch 1. 2-Deoxyribose and hexadecanoic acid were significantly elevated when comparing 1–6% O_2 and threitol or erythritol was shown as statistically different when comparing 1 and 20% O_2 tensions. Erythritol was also detected as a biomarker of heart failure where oxygenation may also be expected to be compromised [22]. These experiments provide new evidence of altered placental metabolism and changes to secreted metabolites in reduced O_2.

Table 3
Metabolite peaks observed to be statistically different when comparing the intracellular extracts detected for O_2 tensions of 1, 6 and 20%

Metabolite	1% O_2 vs 6% O_2	1% O_2 vs 20% O_2
	p-value	*p*-value
Peak 5 unidentified	0.032124571	**0.008809**
Threitol/erythritol (peak 21)	0.058707408	**0.003892**
Peak 22 unidentified	0.028280123	**0.00617**
2-Deoxyribose (33)	**0.008652**	0.016144778
Peak 109 unidentified	0.117185087	**0.00617**

The metabolites highlighted in bold are statistically significant.

The metabolic footprints of placental explant cultures showed a number of differences in these studies. Metabolic footprinting is advantageous as it does not require the technically difficult steps of metabolic quenching or extraction of intracellular metabolites and provides a picture of metabolism over a period of time instead of a snapshot, as is the case for intracellular metabolism. The intracellular metabolome of the explants after culturing was also analysed after extraction. Statistical analyses of this data showed that five metabolites were statistically different including 2-deoxyribose and threitol or erythritol. The identification of fewer discriminatory metabolites and at lower significance, when tissue was compared to conditioned medium samples may result from release of excess metabolites into the extracellular environment, in order to maintain cellular homeostasis.

2-Deoxyribose was elevated in both tissue and conditioned culture medium; this reducing sugar with angiogenic properties [41] has been shown to alter apoptosis and glutathione expression *in vitro* [42]. As 2-deoxyribose was present in higher concentrations in conditioned medium we hypothesise that it was released from cells, either by active transport from living tissue or from dying cells following lysis.

Threitol and erythritol are sugar alcohols derived either from external sources or from the reduction of threose and erythrose and were elevated in conditioned culture medium and the intracellular metabolome from 1% O_2 compared to 20% O_2. Kenny et al. described the elevation of other sugar alcohols, xylitol and ribitol, in maternal serum in PE [43], and erythritol was elevated during heart failure [22]. In addition, Arkwright et al. have described differential expression of small sugars and their derivatives in the syncytiotrophoblast glycocalyx in PE pregnancies [44]. Such increases in sugar alcohols may indicate altered glucose metabolism, which Kay et al. have previously shown to be modified in trophoblast in response to changes in O_2 tension [45]. Hexadecanoic acid was also observed to be elevated in 1% O_2 metabolic footprints compared to 6% O_2. This may be caused by an increased release and conversion of triglycerides and fats for energy usage. We are currently investigating whether the metabolic differences described in this study are present in placental tissue from pregnancies complicated by PE.

Metabolomics is appropriate for investigating changes in cell metabolites present in the conditioned medium and in tissue homogenates. With the use of appropriate methodologies we suggest that metabolomics appears to be a useful tool to investigate the metabolic function of trophoblast or placental tissue and to assess changes in response to altered environmental conditions. In conclusion, metabolomics has the potential to identify novel factors released from the placenta following damaging stimuli, thereby providing new avenues for the investigation of conditions associated with placental damage.

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