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Carbohydrate and fatty acid perturbations in the amniotic fluid of the recipient twin of pregnancies complicated by twin-twin transfusion syndrome in relation to treatment and fetal cardiovascular risk

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Abstract

Introduction: Twin-twin transfusion syndrome (TTTS) complicates 15% of monochorionic twin pregnancies, often being associated with recipient cardiac dysfunction. Untreated, it has a fetal mortality rate of at least 90%; although treatment by fetoscopic laser coagulation significantly improves prognosis. Measurement of recipient amniotic fluid metabolites, such as cardiac Troponin T and atrial natriuretic polypeptide, correlate with cardiac function in this fetus. The aim of this study is to describe the amniotic fluid metabolomic profile in TTTS, relate this to fetal recipient cardiac function and assess the metabolomic changes induced by fetoscopic laser coagulation.

Methods: Prospective single centre cohort study. The metabolomics profile of the amniotic fluid from the recipient sac of TTTS pregnancies was assessed using ultra high performance liquid chromatography-mass spectrometry. Profiles were compared pre- and post-laser coagulation and related to fetal recipient cardiac function, as assessed using Doppler ultrasound within 6 hours of treatment.

RESULTS: Eleven metabolites had significant associations with recipient fetal right and left ventricular myocardial performance index pre-laser. 200 metabolites in recipient amniotic fluid demonstrated a change in relative concentrations when comparing pre- and post-laser coagulation *(p*<0.005*).* The most prominent change is in the balance of carbohydrate and fatty acid metabolic profile contributing to fetal or placental energy metabolism. These changes were also associated with the echocardiographic measures of recipient cardiac function.

Discussion: Changes in carbohydrate and fatty acid metabolic profiles are noted in recipients with cardiac dysfunction, and further changes are noted after treatment.

Validation and investigation may identify targets for potential pharmacological treatment.

Key words: Echocardiography, fetoscopy, laser coagulation, metabolomics, monochorionic twins, twin-twin transfusion syndrome

Abbreviations:

ANF – atrial natriuretic factor

AVA – arteriovenous anastomoses

BNP – brain-type natriuretic factor

DV – ductus venosus

E/A – early passive/atrial

FLC- fetoscopic laser coagulation

IUGR – intrauterine growth restriction

LV – left ventricle

MCDA – monochorionic diamniotic

MPI – myocardial performance index

QC – quality control

RV – right ventricle

TTTS – twin-twin transfusion syndrome

UHPLC-MS - Ultra High Performance Liquid Chromatography-Mass Spectrometry

Introduction

Approximately 15% of monochorionic dichorionic (MCDA) twins are complicated by twin-twin transfusion syndrome (TTTS), a condition associated with unidirectional intertwin blood flow through placental arteriovenous anastomoses (AVA) and high perinatal mortality. This leads to the severe haemodynamic imbalances seen within the fetal circulations of this condition with a hypertensive circulation in the “recipient” twin and subsequent cardiac dysfunction (1), noted in up to 70% of pregnancies. There is corresponding dysregulation of fetal endocrine systems, including the renin-angiotensin-aldosterone (2), atrial natriuretic factor (ANF) (3) and Endothelin-1 systems (4). Such changes have been measured in the amniotic fluid of the recipient twin, and related to cardiac dysfunction and overall fetal prognosis (3).

Untreated, fetal mortality is at least 90%, for each fetus (5). Treatment by fetoscopic laser coagulation (FLC) is effective with survival of two babies approaching 60% (6). FLC also reduces the risk of neurodevelopmental morbidity compared to other treatments (7). Improvement in recipient cardiac function is noted within 48 hours of FLC in approximately half of recipients (8), which is associated with improved fetal outcome (9).

Metabolomics is the holistic untargeted study of metabolism (10) and investigates the final downstream product of genotype-environment interactions. It provides the identification of a dynamic and sensitive phenotypic signature associated with human health ageing and disease molecular pathophysiology (10). In human pregnancy, this technique has been used to investigate intrauterine growth restriction (IUGR) (11-14), pre-eclampsia (15-17) and gestational diabetes (18, 19). In non-pregnant adults, it has been used to profile functional and metabolic changes associated with heart failure (20, 21).

In MCDA pregnancies affected by TTTS, a small number of studies, targeting specific areas of metabolism have been reported (22, 23). Molecular patho-mechanistic changes have been observed in angiogenic growth factors (24), cytokine levels (25) and gene transcripts in amniotic fluid and maternal plasma which appear to predict fetal outcome (26, 27).

We describe the metabolomic profiles in amniotic fluid from the recipient sac of MCDA twin pregnancies complicated by severe TTTS and note associations between fetal echocardiographic recipient right ventricular (RV) and left ventricular (LV) function. In addition, the effects of FLC on the metabolomic profile signatures are described.

Methods

This study had ethical approval from Birmingham Black Country Local Research Ethics Committee (No: 06/Q2702/71 accepted in 2006) with written consent obtained from all subjects.

Patient selection

The cohort consisted of MCDA twins complicated by severe TTTS (presenting before 24 weeks) treated between August 2011 – June 2012 and TTTS was defined as polyhydramnios (>8cm in the deepest vertical pocket of the recipient at <20 weeks of gestation or >10cm from 20 weeks of gestation onwards) in combination with oligohydramnios in the donor (<2cm deepest vertical pool depth). All cases were prospectively staged using the Quintero system (28).

Cardiac function assessment

High-resolution fetal ultrasound and echocardiography were performed in the recipient with curvilinear array transducers (7–3.5 MHz) on a Siemens S3000 ultrasound machine

(Siemens Ltd, Erlangan, Germany) by a single operator (MDK) and the myocardial performance index (MPI) calculated for each ventricle (1, 29) as previously described. Cardiac dysfunction was indicated by the presence of tricuspid regurgitation, reversed flow in the DV during atrial contraction, and a tricuspid early passive/atrial contraction (E/A) ratio of >95% CI outside the normal limits. This was performed within 4 hours of starting the laser procedure and repeated with 6 hours post-FLC.

Fetal cardiac function was assessed 24 hours prior to FLC and repeated within 4 hours post-FLC.

Fetoscopic laser coagulation (FLC)

FLC was performed using local anaesthesia (1% lignocaine skin/myometrial infiltration) and maternal Remifentanil sedation as previously described (30). A selective sequential FLC technique was used, with an additional “Solomon” procedure in some cases (31). No amnio-infusion was performed.

Non-targeted UHPLC-MS metabolomics analysis

All solvents and chemicals applied were of HPLC analytical grade (J.T. Baker, U.K.).

***Sample collection and preparation*.** A 10ml sample of amniotic fluid was taken at insertion of the fetoscope into the recipient amniotic sac and then a further 10ml sample withdrawn at the end of the laser coagulation treatment. The median duration of the laser procedure and amniodrainage the median duration was 34 (range 21 - 45) minutes. Samples were stored at -80℃ before preparation and analysis. All samples were randomised to ensure no correlation between order of preparation and subject, disease grade or date of sample collection. Deproteinisation was performed as described below. 250µL of amniotic fluid was vortex-mixed with 1000µL of methanol for 15 seconds to precipitate proteins and DNA followed by centrifugation (15 minutes, 13,000 *g*) drying to

induce metabolite stability and then stored at -80°C prior to analysis. A pooled quality control (QC) sample was prepared by combining 80µL aliquots of each of the 38 samples (32).

Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) analysis. UHPLC-MS analysis of amniotic fluid extracts and QC samples was performed applying a Dionex U3000 coupled to an electrospray LTQ-FT-MS Ultra mass spectrometer (Thermo Scientific Ltd. UK). Samples were reconstituted in 100µL of 50:50 methanol:water, vortex-mixed for 15 seconds, centrifuged (15 minutes, 13,000 *g*) and transferred to vials with 200µL fixed inserts (Thermo-Fisher Ltd. U.K.). All samples were stored in the autosampler at 5℃ and analysed separately in negative and positive electrospray ionisation (ESI) modes within 72 h of reconstitution. UHPLC separations were performed applying a Hypersil Gold C_{18} reversed phase column (100 x 2.1mm, 1.9µm) at a flow rate of 400µL.min^{-1}, column temperature of 40℃ and with two solvents: solvent A (HPLC grade water + 0.1% formic acid) and solvent B (HPLC grade methanol + 0.1% formic acid). A gradient elution was performed as follows: hold 100% A 0-1.5 min, 100% A - 100% B 1.5-6 min curve 3, hold 100% B 6-12 min, 100% B – 100% A 12-13 min curve 3, hold 100% A 13-15 min. Injection volume was 5µL. UHPLC eluent was introduced directly in to the electrospray LTQ-FT Ultra mass spectrometer with source conditions as follows: spray voltage -4.5 kV (ESI-) and +5 kV (ESI+), sheath gas 30 arbitrary units, aux gas 15 arbitrary units, capillary voltage 35 V, tube lens voltage -100 V (ESI-) and +90 V (ESI+), capillary temperature 280℃, ESI heater temperature 300℃. Data were acquired in the FT mass spectrometer in the *m/z* range 100-1000 at a mass resolution of 50,000 (FWHM defined at *m/z* 400), with a scan speed of 0.4 sec and an AGC setting of 1x10^{6}. Analysis order was composed of 10 QC sample injections for system conditioning followed by a QC sample injection every 6th injection with two QC sample injections at the end of the analytical run.

Amniotic fluid extracts for each subject were analysed in a random order; the two samples for each subject were analysed sequentially.

Data pre-processing. UHPLC-MS raw data profiles were first converted into a NetCDF format within the Xcalibur software's File Converter program. Each NetCDF based three-dimensional data matrix (intensity × *m/z* × retention time – one per sample) was converted into a vector of peak responses, using the freely available XCMS software as described previously (33). Data were exported from XCMS as a .csv file for further data analysis. Metabolite annotation was performed applying the PUTMEDID_LCMS workflow as previously described (34). All metabolite annotations are reported at level 2 (putatively annotated compounds) according to MSI reporting standards (35). In cases where a single metabolite is detected as multiple metabolite features (as described previously (36), only a single feature is reported chosen as having a p-value nearest to 0.05).

Univariate and multivariate analysis. Intergroup comparisons for continuous variables with a non-parametric distribution were made using the Mann-Whitney U test to determine significant differences between the data sets. Median values and 95% confidence intervals (CI) are described. Categorical data were analysed using Fisher's exact test and odds ratios (OR) and 95%CI. Significance was taken as $p<0.05$. Metabolomics processed data were analysed in 'R' applying the unsupervised multivariate principal components analysis (PCA), supervised multivariate Partial Least Squares-Discriminant Analysis (PLS-DA), univariate non-parametric Wilcoxon Signed Rank test and Spearman rank correlation analysis. The fold change (median peak area before treatment/median peak area after treatment) was calculated including 95%CI. Metabolites were manually clustered into classes defining similar chemical structure or metabolic pathway to identify biologically relevant and robust metabolic changes.

Results

Cohort of monochorionic twins complicated by TTTS and the effects of treatment

Table 1 summarises the baseline demographic data of the whole cohort (n=19) at the time of diagnosis. In the total cohort of MCDA twins complicated by TTTS, 5.2% (1/19) had Quintero Stage-I, 15.8% (3/19) Stage-II, and 79% (15/19) Stage-III disease. The measured RV and LV MPI was elevated (>95%CI for gestation) in 89.4% (17/19) and 73.7% (14/19) of recipients, respectively (Figure 1a and b). Of the two cases which had a RV MPI within 95%CI for gestation, one twin set had Quintero stage-I and one Quintero stage-III. In the 5 recipient fetuses with LV MPI within 95%CI, all had Quintero stage >II. In the recipient twin, additional features of cardiac dysfunction are described in Table 1.

FLC was performed at a median gestation of 20 weeks and 2 days (142 days) (95%CI 137.7 – 148.3). Recipient RV MPI (p=0.02) and LV MPI (p=0.03) decreased significantly after FLC (Figure 2a and b).

Amniotic fluid metabolome of the recipient amniotic fluid

38 paired amniotic fluid samples were collected before and after FLC. Following quality assurance of the data, 2694 and 1510 metabolite features remained in positive and negative ion modes, respectively.

Correlation analysis of metabolic profiles and recipient cardiac function in TTTS

Spearman rank correlation analysis was performed to identify associations between recipient RV and LV MPI at the time of diagnosis, and the concentration of each metabolite. A summary of the results demonstrating associations between recipient RV or LV MPI and amniotic fluid metabolites are shown in Table 2 and the full data are shown in

Supplementary Tables 2 and 3. The 118 and 102 metabolites identified as having an association with LV MPI or RV MPI each showed a positive correlation (denoted as '+' showing that as LV MPI or RV MPI increase so does the metabolite concentration) or a negative correlation (denoted as '-' showing that as LV MPI or RV MPI increase the metabolite concentration decreases). 15 metabolites showed an association with LV MPI and RV MPI. Acyl carnitines, acyl glycerides, fatty acids and oxidised fatty acids were all negatively correlated with cardiac function whereas carbohydrates were positively correlated with cardiac function. Ceramides, sphingolipids and glycerophospholipids were also negatively correlated with cardiac function and may be related to changes in cell membranes. Hormones were negatively correlated with cardiac function. Finally, for LV MPI, two oxidative phosphorylation metabolites were negatively correlated with cardiac function but no metabolites were correlated with the RV MPI data.

Comparison of metabolic changes before and after laser treatment independent of type of laser treatment

200 metabolites showed a statistically significant change ($p<0.005$) in relative concentrations when comparing amniotic fluid samples taken before laser, and after laser treatment; this was unaffected by whether or not the Solomon technique was applied. Supplementary Table 1 lists the significantly altered metabolomic profiles (all metabolites are grouped into classes of chemical structure or metabolic pathway). Figure 3 demonstrates these data as a "heat map" of relative concentration changes for all metabolite classes containing three or more metabolites. There were 13 metabolite "classes" consisting of ≥3 metabolites which demonstrated significant fold changes. These include acyl carnitines, acyl glycerides, amino acid metabolism, carbohydrate metabolism,

cholesterol esters, ceramides and sphingolipids, fatty acid metabolism, glycerophospholipids, haem metabolism, nucleosides, oxidised fatty acids, oxidative phosphorylation (electron transport chain) and thyroid/steroid hormone metabolism. These suggest a change in fetal or placental energy metabolism, specifically between carbohydrates (shown by higher levels of carbohydrates before treatment) and fatty acids (shown by higher levels of acyl carnitines, acyl glycerides, fatty acids, oxidised fatty acids and TCA/oxidative phosphorylation metabolites) in amniotic fluid post-FLC. Amniotic fluid oxidised fatty acid concentration shows a 50-fold change (higher concentration post-FLC). This is potentially a prostaglandin derivative known to have vasodilatory roles within the endothelium (37). Of five acyl carnitines, it is the medium chain derivatives (hexanoyl, octanoyl and decanoyl) that predominate. Also changes related to oxidative phosphorylation were noted (Supplementary Table 1). Concentrations of thyroid and steroid hormones were decreased post-FLC potentially related to an attenuated “stress” response post-FLC treatment.

These observed changes may be secondary to placental destruction caused by coagulation (38), changes in recipient cardiovascular function post-laser coagulation or a combination of these events. Finally, uridine/pseudouridine was observed to be 20% higher before treatment and these metabolites have been observed to be increased in heart failure in adults (39).

Discussion

This cohort study demonstrates that the metabolomic profile in the amniotic fluid of recipient monochorionic twins complicated by TTTS is different when there is significant cardiac dysfunction, and that amniotic fluid metabolic profiles change in response to FLC.

Following FLC treatment, a wide range of different metabolite classes are perturbed in amniotic fluid in the recipient sac. The balance between carbohydrates and fatty acid for energy production appears to change following treatment to be preferential for fatty acids. The balance between carbohydrate and fatty acid metabolism appears to be related to recipient cardiac function (as measured by LV and RV MPI) as fatty acids are negatively correlated and carbohydrates are positively correlated with cardiac function. However, it is difficult to delineate whether the source of the metabolites is fetal or placental, as amniotic fluid is a composite of placental metabolite secretion and fetal metabolite secretion (e.g. as urine). As amniotic fluid sampling was repeated after a median time of 34 minutes, it is more probable that these changes are secondary to a combination of trophoblast/vessel destruction (as previously noted by an elevation in alpha-fetoprotein and human chorionic gonadatrophin) (38) and fetal cardiovascular change.

In this relatively small cohort study a significant proportion of the recipient fetuses had evidence of cardiac dysfunction and an elevated LV and RV MPI. Such data are consistent with those previously described in the literature (3) and that recipient RV and LV MPI rapidly alters post-FLC (40). Much of the recipient cardiac effects are due to RV and LV diastolic dysfunction or hypertension (3).

In adults with cardiac failure, prediction of survival is optimal when cardiac ultrasound measurements are combined with serum-derived biomarkers (41). Classically, the combination of cardiac Troponin T, atrial natriuretic polypeptide (ANP) and BNP is used as a marker to reflect cardiac dysfunction and a combination is better than one peptide alone. In recent years there has been a move to a metabolomic approach for profiling functional and metabolic changes in adults with heart failure (21).

In the fetal setting, determination of these peptides within fetal plasma would require fetal blood sampling which would carry significant procedure-related complications. However, therapy for TTTS by FLC involves the removal of redundant amniotic fluid from the recipient's sac after completion of the procedure. At this gestation, amniotic fluid is composed mainly of fetal urine and is therefore a potential source for fetal cardiac biomarkers. The presence of cardiac Troponin T in amniotic fluid has previously been described in severely growth restricted fetuses (42) and also in recipient fetuses with fetal cardiac dysfunction in TTTS (3). Also, in pregnancies complicated by TTTS, ANP and brain-type natriuretic peptide (BNP) have been identified in the amniotic fluid (4, 43) and have also been noted to be associated with fetal recipient cardiac dysfunction in TTTS (3).

15 metabolites in the amniotic fluid of the recipient sac prior to laser coagulation demonstrated an association with LV MPI and RV MPI. Acyl carnitines, acyl glycerides, fatty acids and oxidised fatty acids were all negatively correlated with cardiac function whereas carbohydrates were positively correlated with cardiac function. This implies that the balance between using carbohydrates and fatty acids as substrates for energy metabolism has an influence on fetal cardiac function, though causality still requires testing. This balance has been shown to be important in cardiac diseases including hypertrophic cardiomyopathy (44). Ceramides, sphingolipids and glycerophospholipids were also negatively correlated with cardiac function and may be related to changes in cell membranes. Hormones were negatively correlated with cardiac function. Finally, for LV MPI, two oxidative phosphorylation metabolites were negatively correlated with cardiac function but no metabolites were for the RV MPI data. Of interest and worthy of further study also are the changes in N,N-dimethylarginine and the structurally similar N,N-diacetylspermine. N,N-dimethylarginine is a known inhibitor of NO synthesis from arginine and therefore reduces

vasodilation. Symmetrical and asymmetrical N,N-dimethylarginine are implicated in cardiac function and cardiovascular health (45, 46).

There were also 13 metabolite "classes" consisting of ≥3 metabolites which demonstrated significant fold changes in amniotic fluid after FLC. These include acyl carnitines, acyl glycerides, amino acid metabolism, carbohydrate metabolism, cholesterol esters, ceramides and sphingolipids, fatty acid metabolism, glycerophospholipids, haem metabolism, nucleosides, oxidised fatty acids, oxidative phosphorylation (electron transport chain) and thyroid/steroid hormone metabolism. These suggest a change in fetal or placental energy metabolism, specifically between carbohydrates (shown by higher levels of carbohydrates before treatment) and fatty acids (shown by higher levels of acyl carnitines, acyl glycerides, fatty acids, oxidised fatty acids and TCA/oxidative phosphorylation metabolites) in amniotic fluid post-FLC. Similar changes in the balance of carbohydrate and fatty acid usage for energy production in hypertrophic cardiomyopathy have been previously reported (44). In TTTS, amniotic fluid levels of BNP appear to correlate with the severity of recipient cardiac dysfunction (3). The higher levels of fatty acids and lower levels of carbohydrates post-FLC suggest that there is a switch from fetal or placental use of fatty acids as precursors for energy metabolism to carbohydrates.

Amniotic fluid oxidised fatty acid concentration shows a 50-fold change (higher concentration post-FLC). This is potentially a prostaglandin derivative known to have vasodilatory roles within the endothelium (37). Of five acyl carnitines, it is the medium chain derivatives (hexanoyl, octanoyl and decanoyl) that predominated suggesting a specific perturbation in medium chain fatty acid oxidation.

Two published studies (from the same group) in singleton pregnancies (47, 48) have investigated the amniotic fluid metabolome. These data noted 70 metabolomic compounds

using (1)H NMR. Pregnancies complicated by a heterogeneous group of fetal malformations demonstrated “variations in glucose, some amino acids and organic acids and proteins”. However, it is recognised that this group is heterogeneous for malformations and thus it was difficult to draw conclusions. The amniotic fluid samples were taken by amniocentesis at a wide gestational range (13 – 42 weeks) in these singleton pregnancies. Subjects with gestational diabetes showed an average increase in glucose and small decreases in several amino acids along with acetate, formate, creatinine, and glycerophosphocholine. Small metabolite changes were also observed in the amniotic fluid of singleton pregnancies which eventually underwent preterm delivery and premature rupture of membranes. It is difficult to draw comparisons to our cohort, as these pregnancies have taken amniotic fluid from a recipient sac in complex monochorionic multiple pregnancies (in a narrow gestational age range).

This is the first study of energy metabolism in TTTS and FLC. We have reported the correlation of the balance between fatty acid and carbohydrate use in energy metabolism and their associations with measures of global recipient cardiac dysfunction and the effects of laser ablative treatment. Additional research is required to delineate the origin of these metabolomic changes, although it would be difficult to obtain ethical approval to perform repeat amniocenteses in these high-risk pregnancies which we would enable us to explore these changes. Further targeted metabolomics studies in different biofluids and tissues are now required to identify potential prognostic ‘biomarkers’ to improve outcome in monochorionic twin pregnancies complicated by TTTS.

Contributors: WBD and JWA analysed samples, interpreted data and wrote manuscript. TVM helped with calculation of the MPI and normalising these data as gestationally related

z-scores, as well as contributing to writing the manuscript. RKM, FLM, and CEF helped with procedures, collecting samples, interpreting results and writing manuscript. MDK performed the procedures, interpreted the results and wrote the manuscript. MDK conceived idea. All authors contributed to writing the manuscript and have seen and approved the final version.

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Tables and Figures Legend

Table 1: Demographic and clinical data for all participating subjects (n=19 twin pregnancies; n=38 fetuses)

Table 2: Correlation of metabolic profiles and cardiac function (Left Ventricle (LV) and Right Ventricle (RV) Myocardial Performance Index (MPI)) according to metabolite class

Figure 1: (a) Right ventricular myocardial performance index (RV-MPI) (b) Left ventricular myocardial performance index (LV-MPI) in recipient fetuses

The graph demonstrates individual fetal values against gestational age (and with reference to the 95%CI).

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RV and LV MPI z-scores before and immediately post- fetoscopic laser coagulation (individual data shown).

Figure 3: Heat map showing the distribution of concentrations for individual metabolites (rows) for samples collected before and after fetoscopic laser coagulation (columns). Green shows a low concentration whereas red shows a high concentration in the range of concentrations for each metabolite. Abbreviations are amino acid metabolism (AA), acyl carnitines (AC), acyl glycerides (AG), carbohydrates (C), cholesterol esters (CE), ceramides and sphingolipids (CS), fatty acids (FA), glycerophospholipids (GPL), haem metabolism (H), nucleotides (N), oxidised fatty acids (OFA), oxidative phosphorylation (OP) and thyroid/steroid hormones (TSH).

Supplementary Tables

Supplementary Table 1: Metabolites showing a statistically significant ($p<0.005$) change in relative metabolite concentrations before and after fetoscopic laser coagulation. All metabolites are grouped into classes of chemical structure or metabolic pathway. Fold change is calculated as the median (before treatment)/median (after treatment) and the 95% confidence intervals are included in brackets.

Supplementary Table 2: Metabolites showing an association between their concentration and the Right Ventricle Myocardial Performance Index (MPI) at diagnosis and before fetoscopic laser coagulation. All metabolites listed demonstrated a Spearman Rank correlation between -1.0 to -0.3 or +0.3 to +1.0 and are grouped into classes of chemical structure or metabolic pathway.

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475 **Table 1: Demographic and clinical data for all participating subjects** (n=19 twin
476 pregnancies; n=38 fetuses)

Patient characteristics	**Median (95%CI)**
Maternal age (years)	29 (26.1 – 31.3)
Maternal BMI	24 (22.8 – 27.7)
Gestational age at diagnosis and FLC (days)	142 (137.7 – 148.3)
% difference in EFW	25.2 (21.9 – 31.9)
Recipient fetal cardiac measurements	
Recipient RV MPI (z-score) Median (95%CI)	4.96 (3.62 – 5.50)
Recipient absent/reversed "A-wave" in Ductus Venosus *n* (%)	7/19 (36.8)
Recipient tricuspid regurgitation *n* (%)	15/19 (78.9)
Recipient RV E/A ratio > 95%CI for gestation *n* (%)	11/19 (57.9)
Recipient LV MPI (z-score) Median (95%CI)	2.71 (2.07 – 3.63)
Fetoscopic laser coagulation (FLC) variables	
Duration of FLC and amniodrainage (minutes) Median (range)	34 (21 - 45)
Duration of FLC (minutes) Median (range)	19 (9 – 28)
Number of arteriovenous anastomoses coagulated Median (95%CI)	8 (7.4 – 8.3)
Amniodrainage post-laser coagulation (ml) Median (95%CI)	2600 (1800 – 4000)
Pregnancy outcomes	
Gestational age at delivery (days) Median (95%CI)	227 (207.1 – 232.7)

Perinatal survival of all fetuses* (at 28 days) *n* (%)	30/38 (78.9)
At least one survivor in pregnancy *n* (%)	18/19 (94.7)
Two survivors *n* (%)	12/19 (63.2)
One survivor *n* (%)	6/19 (31.5)
No survivors *n* (%)	1/19 (5.3)

Key: BMI: body mass index, EFW: estimated fetal weight, MPI: myocardial performance index, RV: right ventricle.

(†Intergroup comparisons for continuous variables with a non-parametric distribution were made using the Mann-Whitney U test to determine significant differences between the data sets. For such data, median values and 95%CI are described. Categorical data were analysed using Fisher's exact test and relative risk ratios and 95% confidence intervals. Significance was taken as $P<0.05$ unless otherwise stated)

*Perinatal mortality defined as total number of survivors (all fetuses) who survived until at least 28 days of age.

Table 2: Correlation of metabolic profiles and cardiac function (Left Ventricle (LV) and Right Ventricle (RV) Myocardial Performance Index (MPI)) according to metabolite class

Metabolite Class	Left Ventricle (LV) MPI		Right Ventricle (RV) MPI	
	Number of Metabolites	Correlation coefficient range	Number of Metabolites	Correlation coefficient range
Acyl amino acids	2	+0.34 to +0.39	2	+0.30 to +0.37
Acyl carnitine	5	-0.46 to +0.41	6	-0.43 to -0.31
Acyl glycerides	12	-0.52 to +0.54	9	-0.49 to +0.60
Bile acid metabolism	3	-0.38 to +0.44	3	+0.31 to +0.36
Carbohydrates	3	-0.57 to +0.65	4	-0.33 to +0.45
Ceramides and sphingolipids	8	-0.43 to -0.30	9	-0.49 to +0.42
CoA metabolism	2	-0.34 to +0.36	2	-0.40 to -0.30
Fatty acid metabolism	10	-0.67 to +0.54	13	-0.52 to +0.46
Glycerophospholipids	41	-0.53 to +0.50	28	-0.64 to +0.51
Nucleoside	3	-0.48 to +0.34	4	-0.36 to +0.54
Oxidised fatty acids	4	-0.31 to -0.30	7	-0.48 to +0.57

The metabolites identified as having an association with LV MPI or RV MPI (denoted as '+' correlation coefficient shows that as LV MPI or RV MPI increase so does the metabolite concentration) or a negative correlation (denoted as '-' shows that as LV MPI or RV MPI increase the metabolite concentration decreases).

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The graph demonstrates individual fetal values against gestational age (and with reference to the 95%CI).

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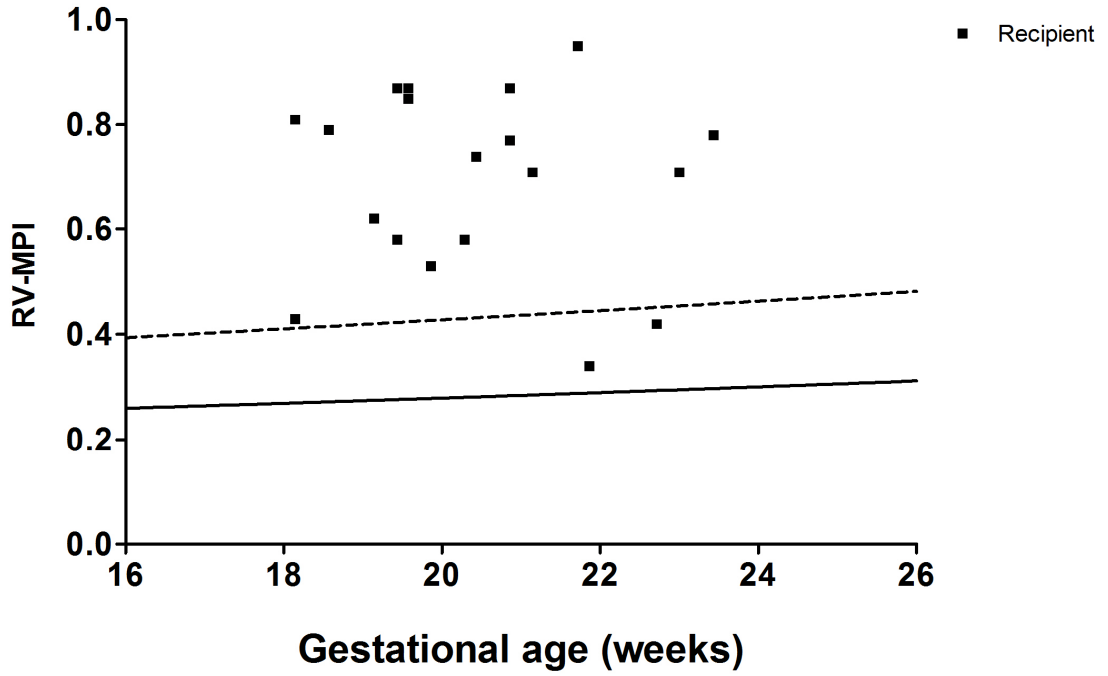
The graph demonstrates individual fetal values against gestational age (and with reference to the 95%CI).

(a)



(b)

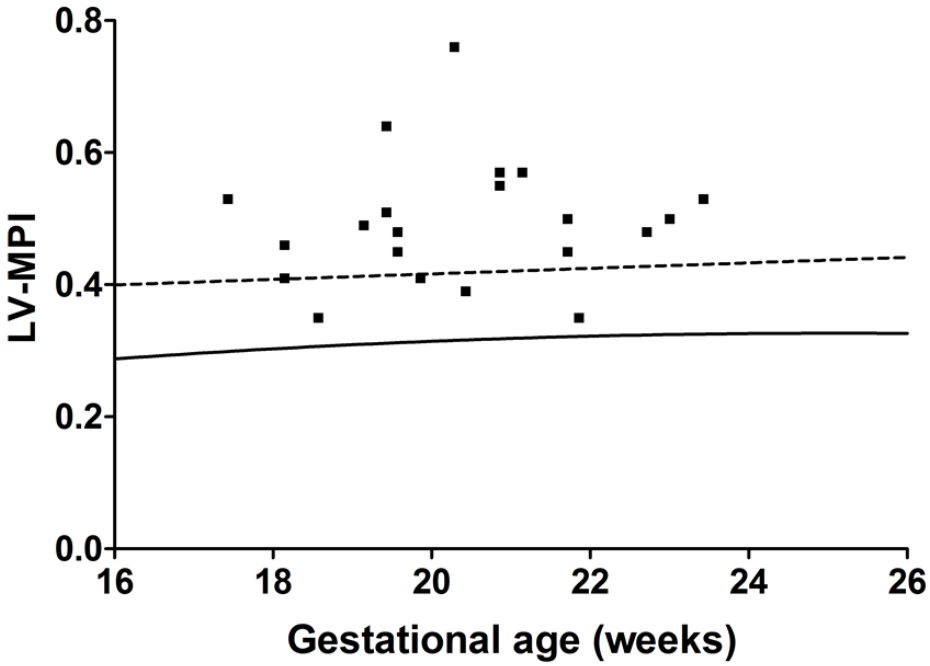
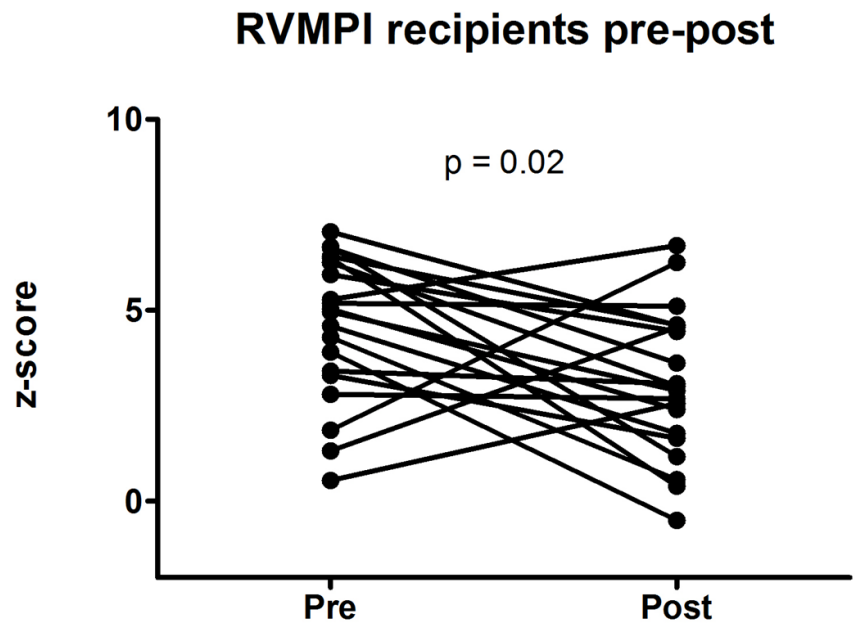


Figure 2: Changes in: a) Right Ventricular Myocardial Performance Index (RVMPI) and b) Left Ventricular Myocardial Performance Index (LVMPI) after pre- and post- fetoscopic laser coagulation in recipient twins

RV and LV MPI z-scores before and 6 hours post- fetoscopic laser coagulation (individual data shown).

a)



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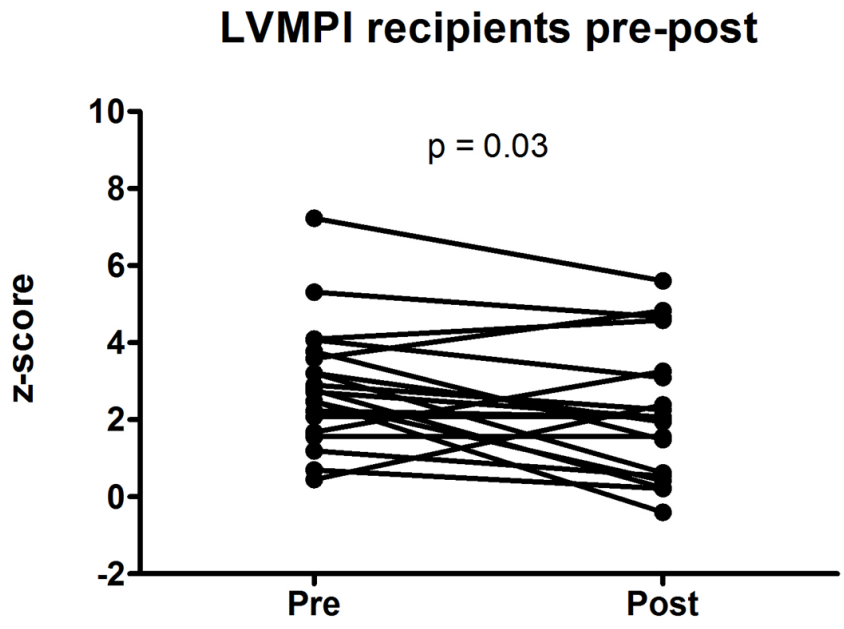

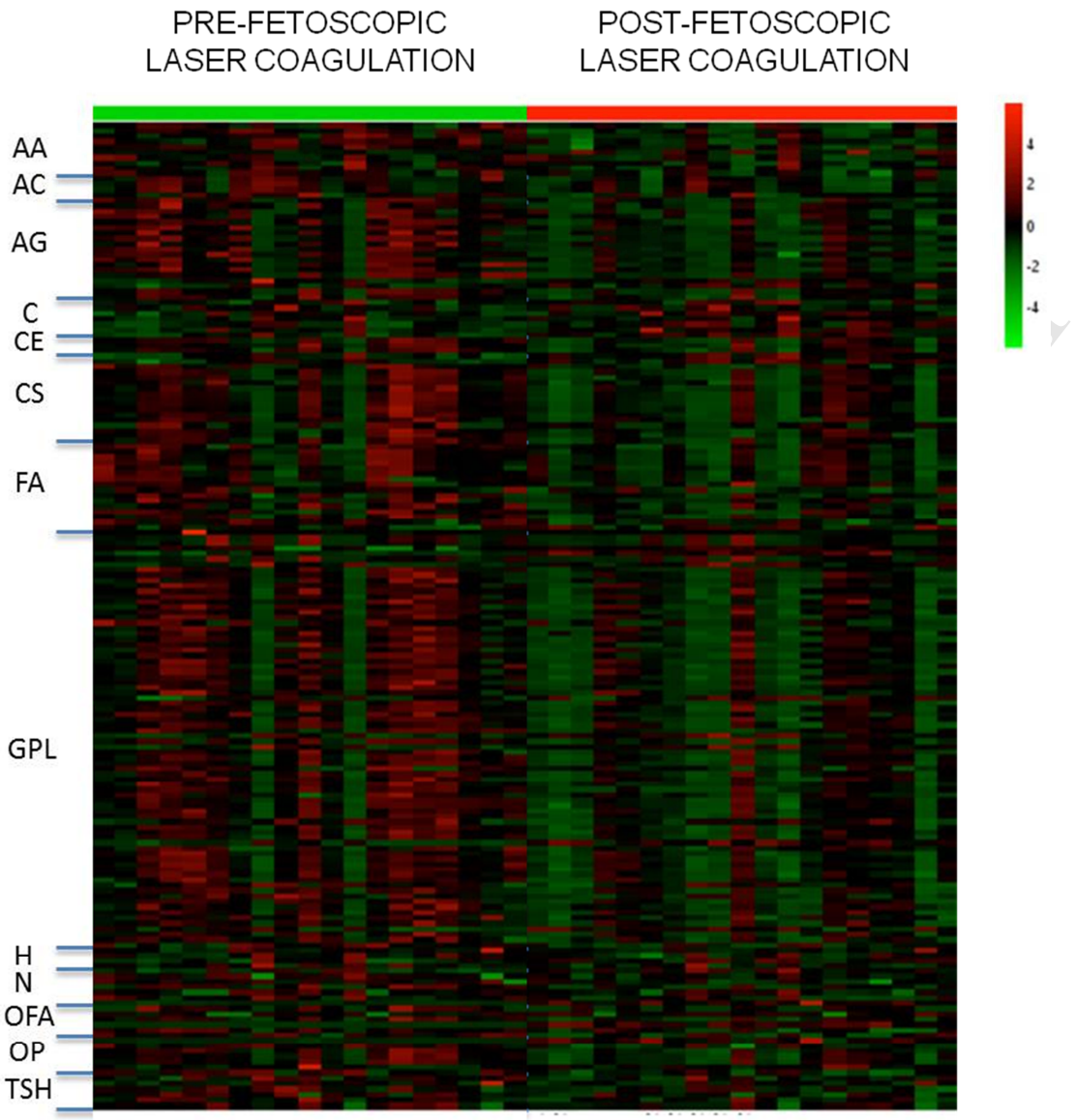
PRE-FETOSCOPIC
LASER COAGULATION
POST-FETOSCOPIC
LASER COAGULATION
AA
AC
AG
C
CE
CS
FA
GPL
H
N
OFA
OP
TSH
4
2
0
-2
-4

Highlights

1. Metabolomic changes are seen after fetoscopic laser coagulation (FLC)
2. Carbohydrate and fatty acid metabolism appears altered following FLC
3. These findings are in keeping with changes seen in adults with cardiomyopathy
4. Metabolomics may provide new biomarkers for twin twin transfusion syndrome