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Article

Acute Fetal Metabolomic Changes in Twins Undergoing Fetoscopic Surgery for Twin-Twin Transfusion Syndrome

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Abstract

Fetuses undergo major surgical stress as well as fluid shifts secondary to both twin-twin transfusion (TTTS) as well as the fetoscopic surgery for treatment of TTTS. While the pathophysiology of TTTS is understood, the acute metabolic changes that fetuses experience from fetoscopic surgery are not. We sought to evaluate the changes in recipient metabolomic profile secondary to TTTS surgery. Amniotic fluid was collected at the beginning and end of four TTTS surgical cases performed from 12/2022−2/2023. Samples were immediately processed and evaluated via NMR-based Metabolomics Facility protocol. In univariate analysis, 12 metabolites (glucose, lactate, and 10 key amino acids) showed statistically significant changes between the beginning and end of the surgery. Among these, 11 metabolites decreased at the end, while only lactate increased. Supervised oPLS-DA modeling revealed pyruvate and lactate as the two metabolites most impact on the variance between cases, and that 40% of metabolomic changes could be attributed directly to the timing that the sample was taken (i.e., if pre- or postoperatively). These results indicate significant metabolic changes in the recipient twin during fetoscopic surgery for TTTS. These findings of decreased glucose, increased lactate, and decreased amnio acids would indicate increased catabolism during surgery. This study raises questions regarding optimal maternal and fetal nutrition during surgery and if nutritional status could be optimized to further improve twin survival during fetoscopic surgery.

Keywords: twin-twin transfusion; monochorionicity; metabolomics

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Twin-twin transfusion syndrome (TTTS) is a disease that complicates up to 15% of monozygotic twin pregnancies (Society for Maternal-Fetal Medicine & Simpson, 2013; Ville et al., 1995). The pathophysiology of the disease is due to imbalances in vascular connections on the placental surface, leading to a hypovolemic (donor) twin shunting blood volume to a resultant hypervolemic (recipient) twin. When TTTS presents in the second trimester and progresses beyond early-stage disease, there is as high as a 90% mortality rate and an 18% risk of neurodevelopmental complications of survivors in pregnancies managed expectantly (Haverkamp et al., 2001; Lopriore et al., 2009). Fetoscopic laser photocoagulation (FLP) of these shared placental vascular connections is the principal management of TTTS, as interruption of the connections between the two fetuses creates a functional dichorionic placenta (Senat et al., 2004; Society for Maternal-Fetal Medicine & Simpson, 2013).

Understanding fetal metabolic status has been a significant challenge due to the inability to directly test the fetal blood without invasive procedures that carry significant risks to the fetus or pregnancy. This has led to the use of amniotic fluid metabolomics to assess the fetal nutritional status (Moco & Buescher, 2023). Metabolomics is the study of small intermediates and/or products of cellular metabolism that can acutely reflect the fetal status in various disease states (Chen et al., 2023). Previous work in the field of metabolomics has shown that TTTS and the resultant cardiac strain in recipient twins leads to alterations in fatty acid, glucose, and steroid hormone metabolism (Parchem et al., 2023). What is not known, however, is what the impact of surgery itself is upon the fetus, especially in fetuses with cardiac strain. The high mortality rate of untreated TTTS is well-established, but even with optimal surgical management, the overall survival rate remains below 85% (Society for Maternal-Fetal Medicine & Simpson, 2013). Given the continued risk of fetal death with laser surgery even with optimal treatment as well as that the field of fetal surgery continues to expand for treatment of additional fetal conditions, understanding the metabolic alterations, if any, during surgery itself is paramount as it may provide insight into the fetal stress during cases, and provide an opportunity for perioperative fetal nutritional supplementation to allow them to better tolerate surgery and improve outcomes.

Thus, we sought to evaluate the fetal metabolomic profile immediately pre- and postoperatively from FLP for treatment

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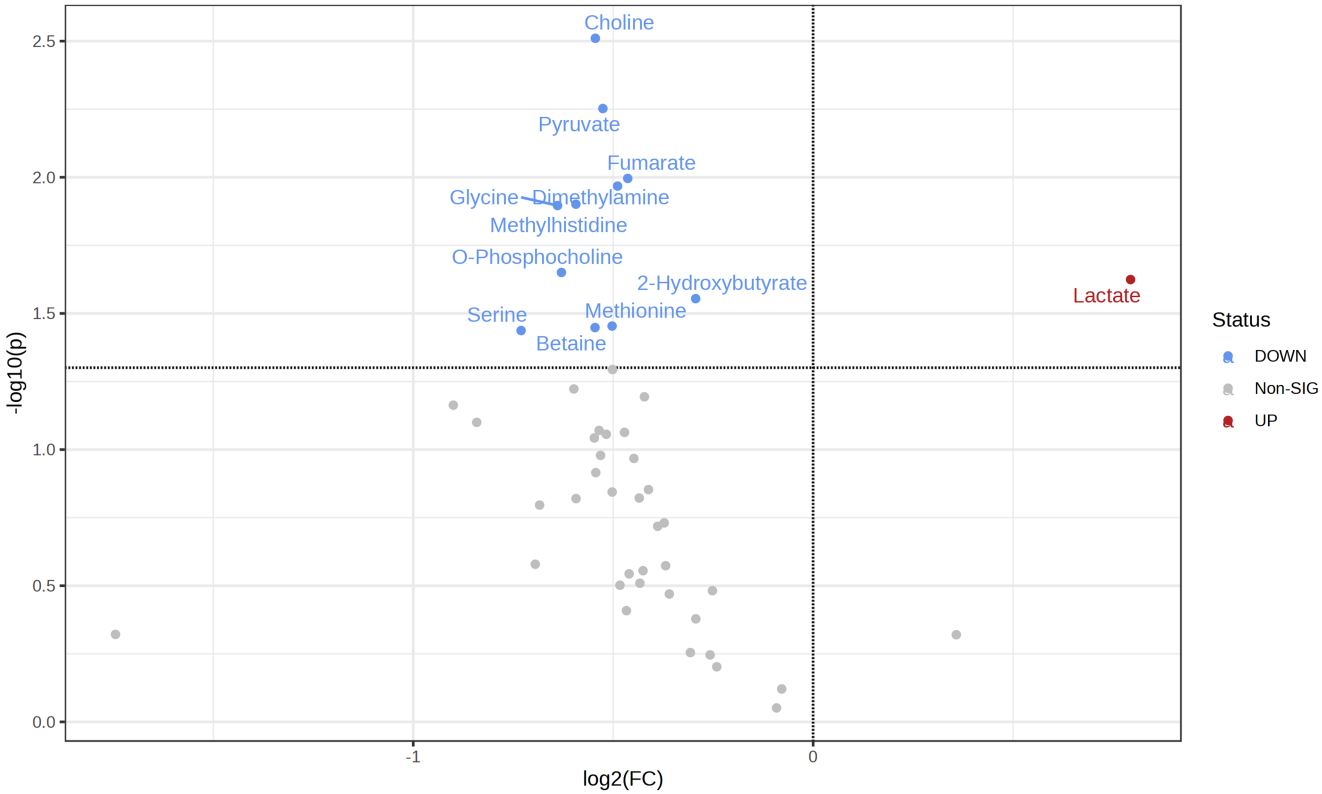

Figure 1. Volcano plot of log changes pre and post operative metabolites. Lactate was the only metabolite increased from start to end of the case. Statistically significant decreased metabolites are listed in Table 1.

of TTTS to assess whether there were changes in recipient twin metabolomic profiling from the surgery to garner a better understanding of the stress of surgery upon the fetus.

Methods

This was a subanalysis of an ongoing institutional review board (IRB) approved prospective cohort study (Cincinnati Children's Hospital Medical Center IRB #2017-2414) in which amniotic fluid that would otherwise be discarded at the time of fetal surgery can be collected for research and analyzed. Patients must sign informed consent with approved study staff prior to surgery to be enrolled in the study. Amniotic fluid was collected at the beginning and ending of fetoscopic surgical cases performed for treatment of twin-twin transfusion surgery. Specifically, the amniotic fluid was removed and processed at the beginning of the case immediately after scope insertion and after conclusion of all lasering/remapping, but prior to amnioreduction, the fluid was removed. In an attempt to homogenize the population being evaluated, samples from cases between 18−22 weeks were selected and cases with either fetal hydrops (i.e., stage IV TTTS) or stage 3 recipient cardiomyopathy (defined as left ventricular myocardial perfusion index ≥ 0.53 or right ventricular myocardial perfusion index ≥ 0.64) were excluded as there was concern that fetuses with severely compromised cardiac function may no longer possess the same adaptive processes during surgical stress as other fetuses.

All metabolomics analysis was performed at NMR-Based Metabolomics Facility (RRID: SCR_022636). The samples were filtered at 12,000x g_n for 90 min at 4°C using prewashed 3 kDa spin filters (NANOSEP 3K, Pall Life Sciences). The NMR sample was prepared by mixing filtrate with NMR buffer (100 mM phosphate buffer in D_2O, pH 7.3, and 1.0 mM TMSP (3-Trimethylsilyl 2,2,3,3-d_4 propionate) up to the final volume of 600uL. All NMR spectra were acquired on a Bruker Avance II 600 MHz spectrometer with BBO Prodigy probe. Chemical shifts were assigned to metabolites based on reference spectra found in databases, Human Metabolome Database (HMDB; Wishart et al., 2007), and Chenomx® NMR Suite profiling software (Chenomx Inc. version 8.4). The assigned metabolites were quantified using Chenomx software based on the internal standard, TMSP. All the metabolomics data analysis were performed using R studio and MetaboAnalyst 5.0 (Pang et al., 2021).

Results

Four samples were collected between 12/2022−2/2023. The gestational ages were 18w4d, 21w4d, 21w4d, 21w2d, respectively of the samples. All cases were either stage II or III TTTS, and the recipients all had similar myocardial perfusion indices (all left ventricular myocardial perfusion indices < 0.53 and right ventricular myocardial perfusion indices < 0.64). In the univariate analysis, 12 metabolites showed statistically significant changes between the beginning and end of the surgery. Among these, 11 metabolites decreased at the end, while lactate increased. The overall trend revealed an alteration in glucose, lactate and amino acid metabolism, with increased amniotic fluid lactate, and decreased amniotic fluid glucose as well as key amnio-acids. The volcano plot revealed the fold change (FC) and p values for each metabolite (Figure 1, Table 1). The metabolites with significant changes in the univariate analysis included choline, pyruvate, fumarate, dimethylamine,

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Table 1. Fold change of significantly different metabolites pre and post operatively.

	Fold change	*p* value
Choline	0.686	.003
Pyruvate	0.695	.006
Fumarate	0.725	.010
Dimethylamine	0.713	.011
Methylhistidine	0.663	.013
Glycine	0.642	.013
O-Phosphocholine	0.646	.022
Lactate	1.734	.024
2-Hydroxybutyrate	0.816	.028
Methionine	0.706	.035
Betaine	0.685	.036
Serine	0.603	.037

methylhistidine, glycine, O-phosphocholine, lactate, 2-hydroxybutyrate, methionine, betaine, and serine (Table 1).

The *t*-test (unpaired) analysis also identified significant differences between before and end of surgery for several metabolites, including 2-hydroxybutyrate, betaine, choline, dimethylamine, fumarate, glycine, lactate, methionine, methylhistidine, o-phosphocholine, pyruvate, and serine. In the paired analysis, several metabolites showed significant changes between before and end of surgery. These metabolites included 2-hydroxybutyrate, 2-oxoisocaproate, 3-hydroxybutyrate, alanine, betaine, choline, citrate, creatine, creatinine, dimethylamine, ethylmalonate, formate, fumarate, glucose, glutamine, glycine, isobutyrate, isoleucine, lactate, leucine, lysine, methylhistidine, myo-inositol, N,N-dimethylglycine, o-phosphocholine, ornithine, proline, pyruvate, serine, sn-glycero-3-phosphocholine, threonine, trans-4-hydroxy-L-proline, tryptophan, tyrosine, and valine. As part of the univariate analysis, fold changes for the individual cases were evaluated, and trends were similar among patients (Figure 2a and 2b).

When analyzing the pre- and postoperative metabolites via principal component analysis (PCA), 95% of the variance was explained by the timing of sample collection (i.e., was the sample pre- or postoperative), in the PCA model (Figure 3a). This was also evident on hierarchical clustering heatmap, which revealed that

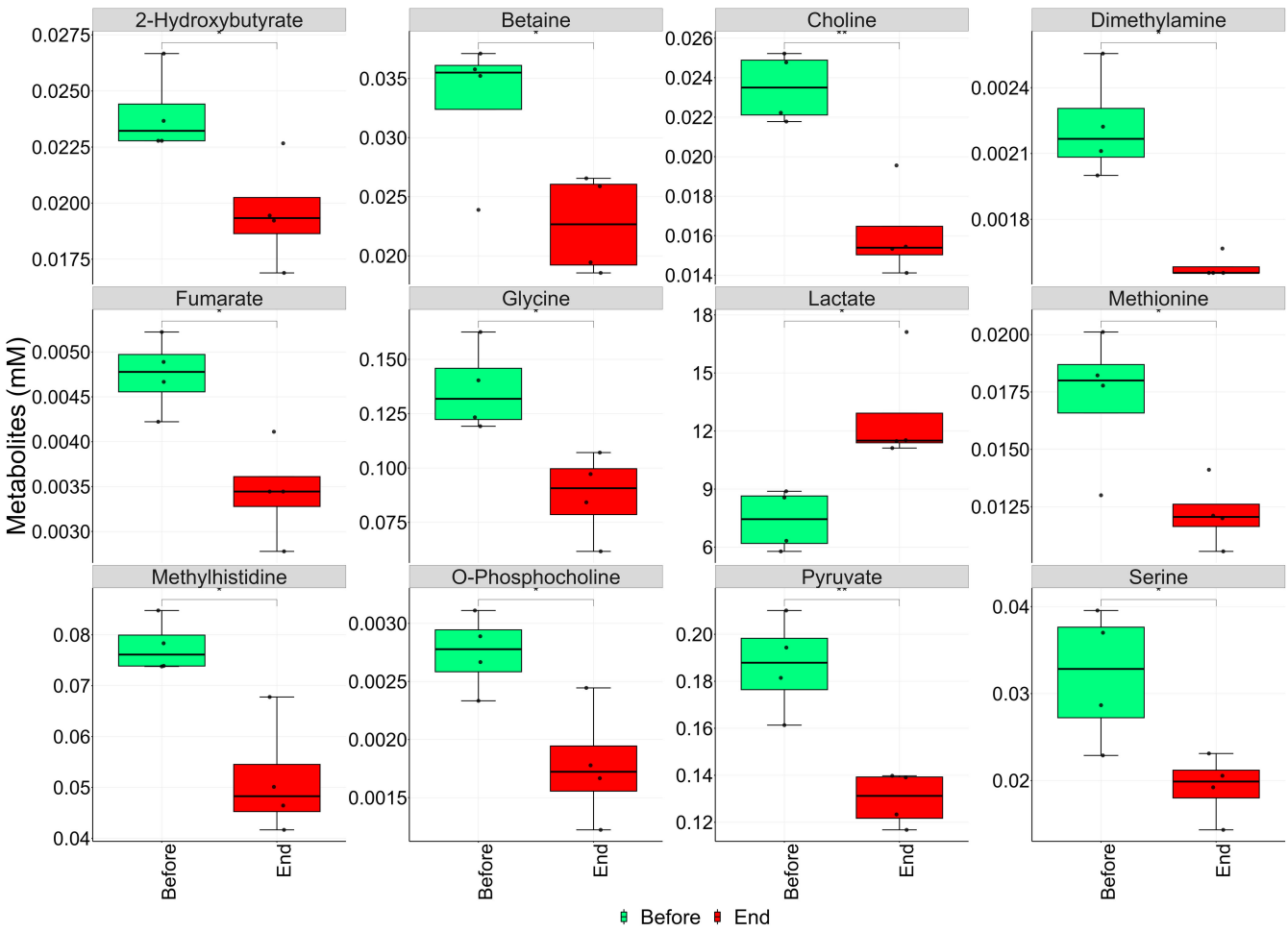

Figure 2a. Box and whisker plots of statistically significant different metabolites pre and post-operatively.

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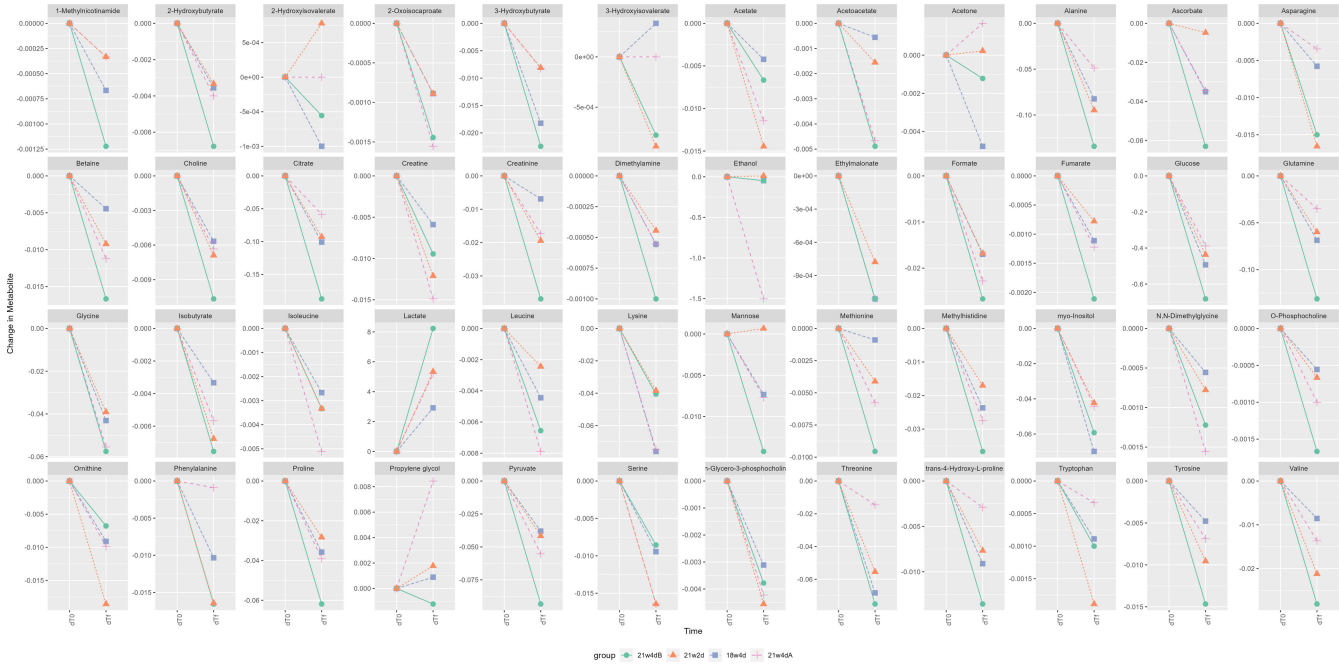

Figure 2b. Metabolite change with each surgical case.

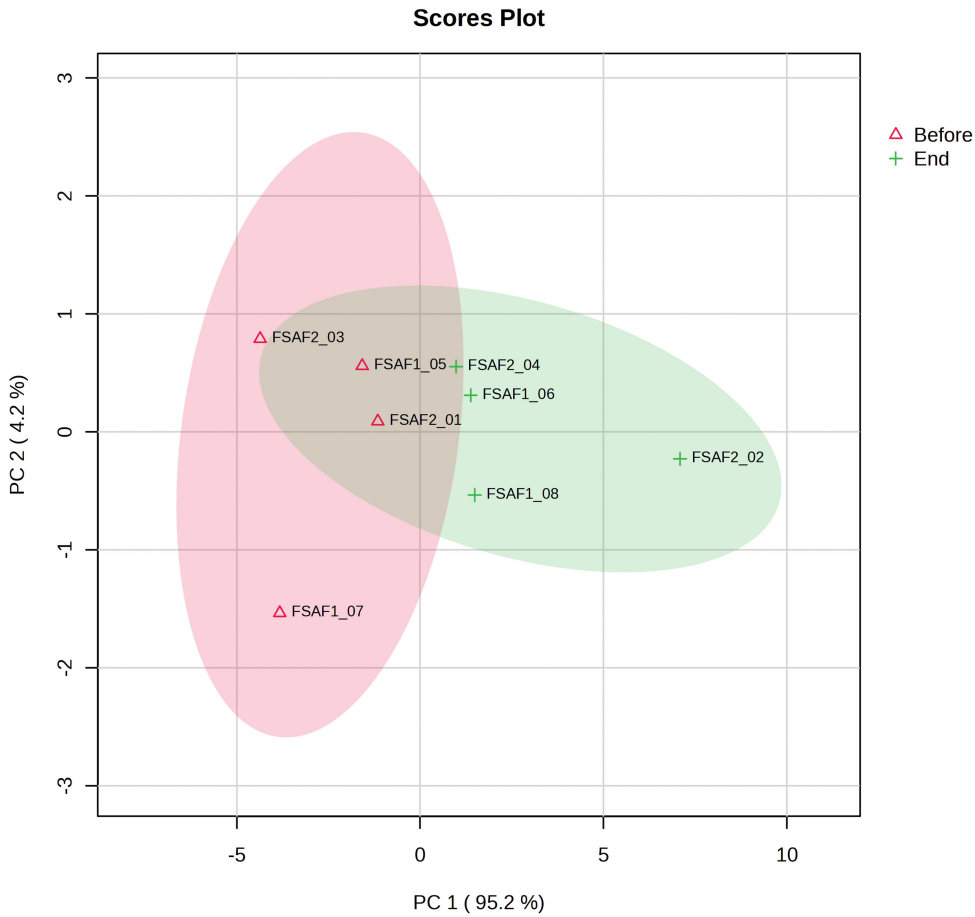

Figure 3a. Principal component analysis scores plot. Principal component 1 was being pre- or postoperative. Principal component two was the sample number. On principal component analysis, 95.2% of variance in metabolite could be ascribed to the timing of the sample (i.e., pre- vs. postoperative).

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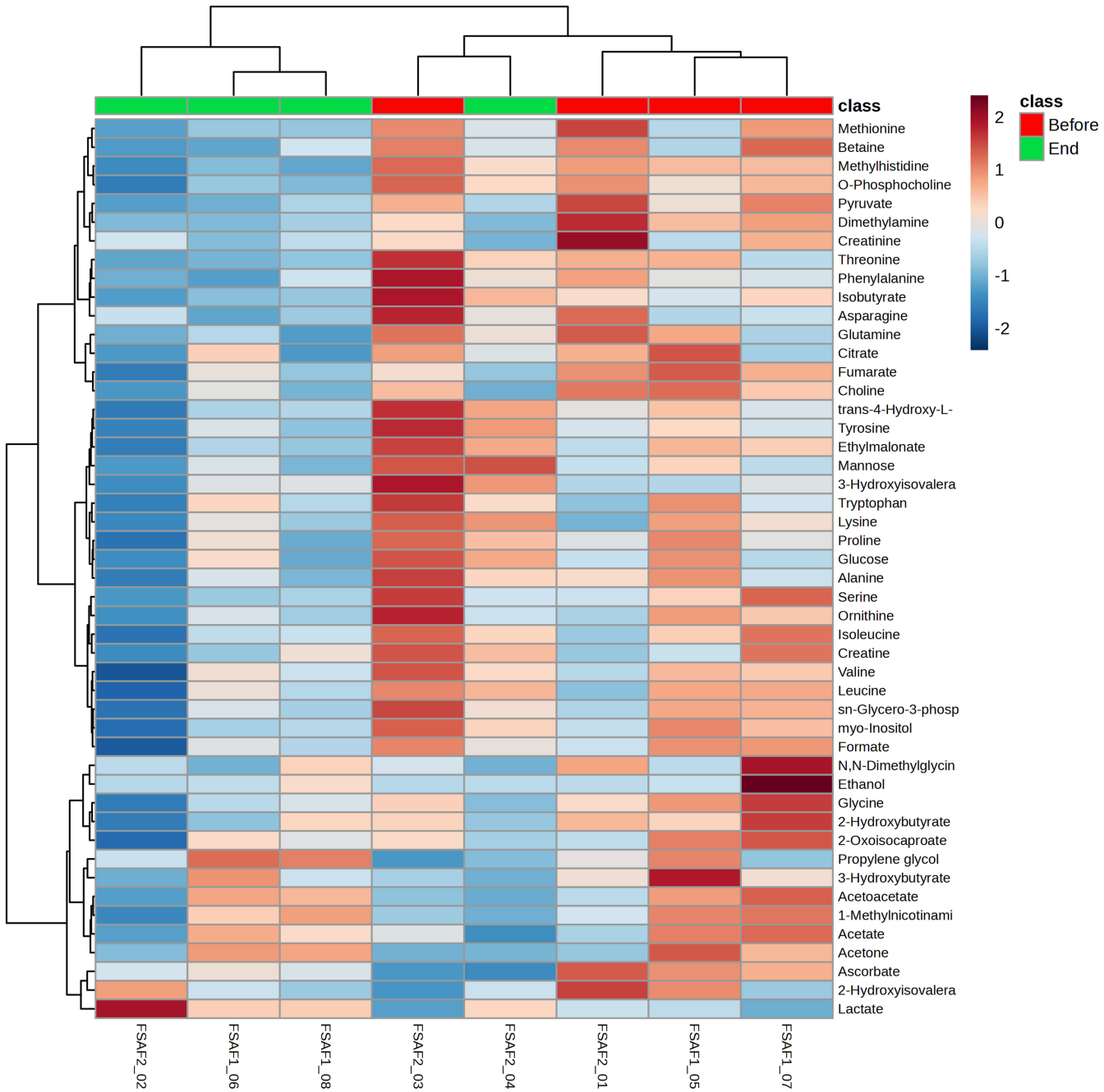

Figure 3b. This heatmap visualizes the relative concentrations of 48 metabolites in samples taken before and after surgery. Each row represents a distinct metabolite, while columns denote individual samples. Hierarchical clustering, using Ward's method on a Euclidean distance measure, was applied to group metabolites based on their similarity patterns across the samples. The dendrogram on the left illustrates the clustering results, where branches represent relationships between metabolites based on their concentration profiles.

although there were significant differences in the absolute metabolite levels between cases, the pre- to post-trend was similar between cases (Figure 3b). When evaluating via supervised orthogonal partial least squares-discriminant analysis (oPLS-DA), the pre- and post-operative groups were distinctly different and this was statistically significant. In oPLS-DA modeling, being pre- or postoperative explained 40% of the variance in metabolites (Figure 4a). The variance of metabolomic profiles within cases was up to 30% and was not significantly different between cases (Figure 4a). The loadings of oPLS-DA of the pre- and postoperative metabolites revealed that while many amnio-acids, as well as lactate, played a significant role in the model; lactate had the highest variance both intracase (before and after) and intercase (all before samples and all after samples, Figure 4a and 4b). The metabolites with the highest VIP scores, or those most impactful in the model were (in descending order) pyruvate, dimethlamine, choline, fumarate and lactate, with VIP score of at least 1.4 for all aforementioned metabolites (Figure 4b). Glycine, a key amnio-acid player in metabolic processes, was just below a VIP score of 1.4. Pyruvate is a known precursor to lactate production and the pyruvate depletion, as well as its highest VIP score, may be reflected in the elevated lactate level as well.

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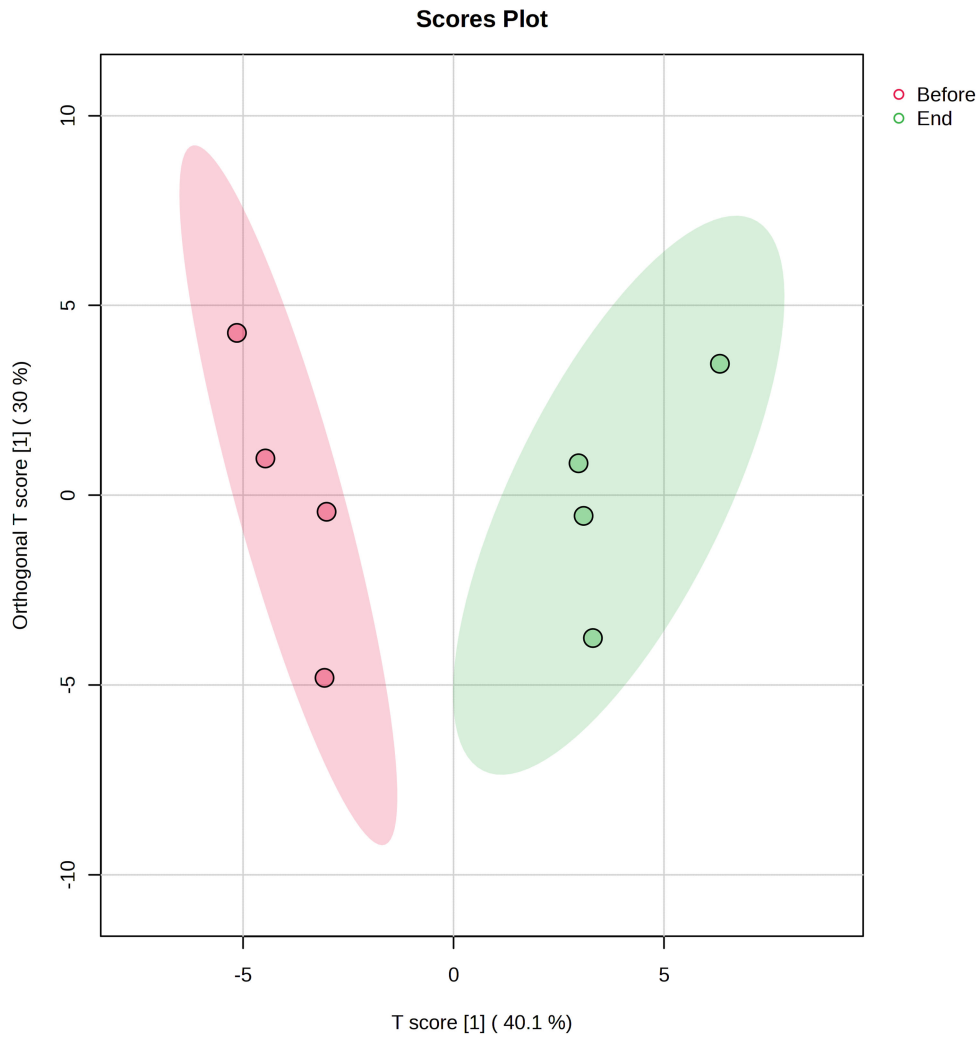

Figure 4a. Supervised oPLS-DA scores plot. The plot represents an oPLS-DA analysis with samples taken before (red circles) and at the end (green circles) of surgery. Each dot corresponds to an individual sample's metabolic profile. The shaded regions depict the 95% confidence intervals for each group. The *t* score on the x-axis indicates that approximately 40.1% of the variance in the data is attributed to the timing of the sample (i.e., pre- vs. postoperative).

Discussion

Metabolomic profiling of recipient twin amniotic fluid pre- and postoperatively revealed alterations in glucose, lactate and amino acid metabolism, with increased amniotic fluid lactate, and decreased amniotic fluid glucose as well as key amnio-acids. This would imply a depletion of normal energy usage pathways and use of alternative metabolic pathways for fetal energy as glucose, lactate and amnio-acids are all major sources of fetal nutrition (Kyllo et al., 2023). In states of impaired fetal oxygenation, such as placental insufficiency, the consumption of these energy sources change, with an overall decrease in glucose and an increase in lactate (Brown et al., 2022; Cetin et al., 2020; Thorn et al., 2013). Further, in states of fetal hypoxemia or significant stress, there is a consumption of available glucose. This is followed by pyruvate production secondary to glycolysis, and that pyruvate is converted to lactate via the placenta or fetus to increase the fetal lactate as an energy source (Char & Creasy, 1976; Jones et al., 2021). It thus is quite biologically plausible that the surgical stress of FLP and the major fluid shifts occurring during the lasering of the placenta would lead to increased consumption of alternative energy sources for the fetus, and the metabolomic profiling in this study captures that acute strain on fetal metabolism. Other high VIP score metabolites, such as choline, are known to play key roles in proper fetal growth and alterations in fetal glucose lead to abnormalities in choline metabolism (Zhou et al., 2021).

While amniotic fluid lactate has not been scrutinized during fetal surgery, there has been study of amniotic fluid lactate during labor and specifically abnormal labor. It appears that the amniotic fluid is used as a reserve for lactate, which the placenta and fetus can deposit lactate for further use when undergoing periods of significant stress, such as with labor (Akerud et al., 2009; Wiberg-Itzel, 2022). It thus again stands to reason that the increased amniotic fluid lactate may be a reflection of both the fetal and uterine stress from the fetoscopic surgery. Lactate is also know to decrease pH, and given the concerns for alterations in amniotic fluid pH leading to amniotic membrane damage (Forde et al., 2023), one must wonder if a longer or more stressful surgery for a fetus would correlate to an increased risk of adverse outcomes postoperatively. Further study into the amniotic fluid lactate and long-term outcomes is warranted.

This study raises the question of how aerobic metabolism and fetal oxygenation might be improved during FLP and if that was successful, could that lead to improved postoperative outcomes? Furthermore, the fetal glucose is directly related to the maternal glucose (Ornoy et al., 2021), and in our clinical practice, mothers

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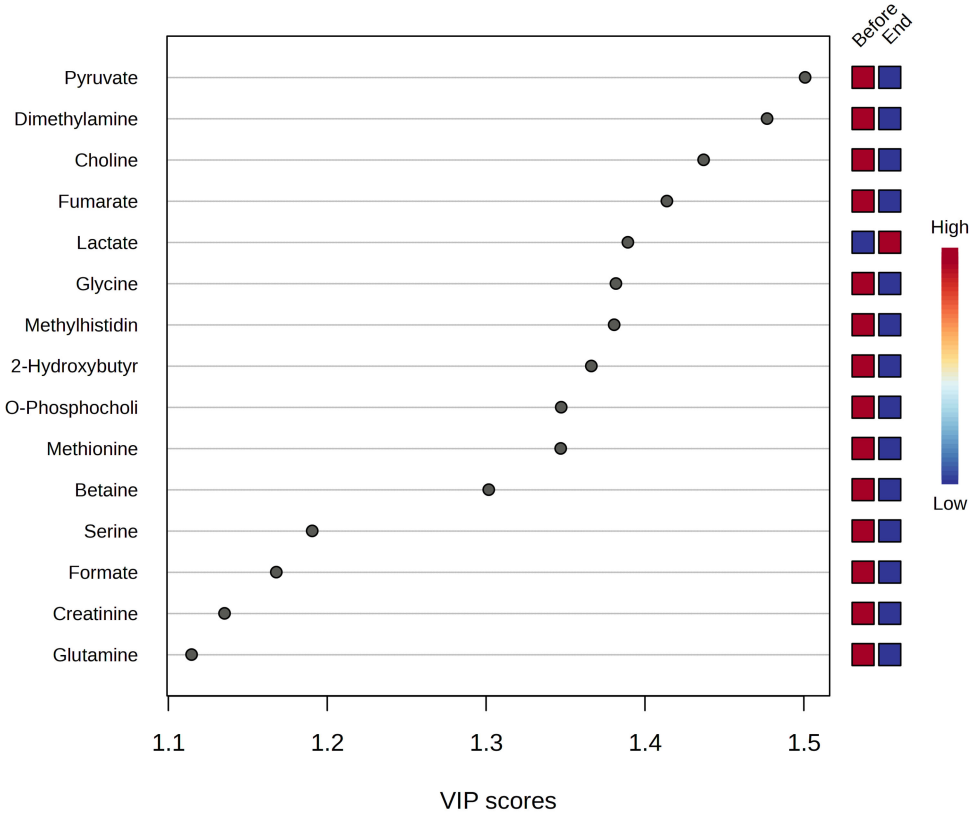

Figure 4b. VIP scores of the various metabolites that changed pre- and postoperatively. A VIP score > 1 is considered to be significant. Pyruvate had the highest VIP score of 1.5, possibly attributing to the significant decrease in pyruvate pre- and postoperatively, as well as its role in lactate production. Key players in energy usage and metabolism, such as glycine and lactate, had high VIP scores as well.

are fasting for 8 hours prior to surgery due to the concern for airway protection for these patients. However, only a minute fraction (<1%) of patients will require general anesthesia for fetoscopy. Eating during labor, and certainly liquid consumption, has been shown to be safe and not negatively impact maternal outcomes, even with the risk of an emergent cesarean, which carries a higher risk of intubation than fetoscopic surgery (ACOG Committee Opinion No. 766, 2019). Is is possible that by decreasing the time mothers are forced to remain NPO, their glucose levels will be higher, their serum lactate lower, and the fetuses then do better with surgery? This is something that warrants further evaluation.

A strength of this study is the novelty and biologic plausibility of the findings, as well the the key clinical implications of the findings (as detailed above). A weakness of this study is the low *N*, and larger studies are both planned and warranted to validate or refute the findings of this work. Additionally, it is important to consider that during fetoscopic surgery, sometimes an amnioinfusion is required to properly perform the surgery. On average in these four cases, approximately 450 cc of IV fluid (i.e., Ringer's Lactate) was used. Typically about 200 cc of fluid is wasted and/or is in the IV tubing, therefore approximately 250 cc of LR, which of course contains lactate in it, was infused during the cases. These amnioinfusions occur prior to fluid removal (i.e., performance of amnioreduction to normalize the amniotic fluid at the end of the case), thus 250 cc would represent a small fraction of the amnioinfusion volume, and the one case that did not perform an amnioinfusion at all had a significantly increased lactate relative to the start of the case and a closing lactate equivalent to cases where amnioinfusion was performed. Another limitation of the study is the possibility of heterogeneity in the samples by stage of disease. Supporting homogeneity in the samples is that both principal component analysis did not identify stage of TTTS as impactful upon the metabolomic changes, and all recipients had myocardial perfusion index scores less than 0.53 and 0.64 for the left and right ventricle respectively; however given that both stage II and stage III disease was included, some degree of difference secondary to staging is possible.

Conclusion

Amniotic fluid metabolomic profiling pre- and postoperatively after fetal surgery for TTTS reveals alterations in normal metabolic pathways; specifically, decreased amnio-acids and glucose, and increased lactate. Further work is needed to understand the impact of these changes on pregnancy and neonatal outcomes, and fetal metabolic optimization may be an area for future improvement during management of complex twins and TTTS.

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Data availability statement. The data supporting the findings of this study are included within the manuscript. For any inquiries regarding de-identified metabolite profiling or other data, please contact the corresponding author.

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Ethical statement. Informed consent was obtained from all participating patients in accordance with the Institutional review board of Cincinnati Children's Hospital. All procedures involving human participants were in accordance with the ethical standards of the institution and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards.

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