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Twin-Twin Transfusion Syndrome Is Associated with Alterations in the Metabolic Profile of Maternal Plasma in Early Gestation: A Pilot Study

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The authors declare that they have no competing interests.

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Bulleted statements:

What's already known about this topic?

- Twin-twin transfusion syndrome (TTTS) is associated with fetal and perinatal mortality and early intervention by fetoscopic laser coagulation of the placental arteriovenous anastomoses may increase the chance of a favorable outcome and reduce morbidity.
- Maternal circulating "biomarkers" for aiding the prediction of TTTS in the first trimester are still lacking.

What does this study add?

- Based on a large prospective longitudinal twin birth cohort, we used a prospectively collected case-controlled cohort of monochorionic twins that developed TTTS, an altered metabolome of maternal plasma before16 weeks was noted. This principally involved fatty acids, TCA cycle intermediates and amino acids those involved in angiogenesis and energy metabolism.
- Metabolic perturbations can be detected in maternal plasma before ultrasound features that are diagnostic of TTTS, and could therefore be a promising approach

for early screening of monochorionic twin pregnancies.

Availability of data and materials

The LOTiS datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abstract

Objective: Twin-twin transfusion syndrome (TTTS) causes perinatal mortality and morbidity in monochorionic (MC) twins. Early recognition and interventional therapy for TTTS is associated with a more favorable overall prognosis. However, the prediction by ultrasound in the first trimester has relatively poor sensitivity and specificity. This study aims to profile maternal metabolic changes before the clinical onset of TTTS and identify potential metabolic biomarkers to aid ultrasound screening.

Method: Maternal plasma was prospectively collected between 11-15 weeks of gestation in apparently uncomplicated MCDA pregnancies. This cohort was divided into: i) patients subsequently diagnosed using ultrasound with TTTS and ii) uncomplicated matched controls. Gas chromatography-mass spectrometry was used for metabolomic profiling.

Results: The levels of fatty acids, organic acids, oxaloacetic acid, and beta-alanine were significantly lower in the maternal plasma of TTTS at 11-15 weeks of gestation, while methionine and glycine were higher ($p<0.05$, FDR<0.12). Generally, in TTTS pregnancies, metabolism of amino acid, carbohydrate, cofactors, vitamins, and purine were ‘down-regulated’; whilst bile secretion and pyrimidine metabolism were ‘upregulated’.

Conclusions: Metabolomics scanning of early gestation maternal plasma may identify those pregnancies that subsequently develop TTTS, especially,

downregulated fatty acid level may be biologically plausible to be implicated in the pathogenesis of TTTS.

Keywords: Twin-twin transfusion syndrome, GC-MS, metabolite, biomarker

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1. Introduction

Twin-twin transfusion syndrome (TTTS) complicates 15% of monochorionic diamniotic (MCDA) twin pregnancies and the underlying etiology and predisposition is the presence of unidirectional placental arteriovenous anastomoses causing imbalanced intertwin hemodynamics[1,2]. This leads to the "recipient fetus" having a "*hyperdynamic*" circulation with increased cardiac afterload with increased ventricular and systemic blood pressure[3], and secondary endocrine dysfunction, involving the renin-angiotensin-aldosterone, atrial natriuretic factor and endothelin-1 systems[4]. In contrast, the "donor fetus" rarely demonstrates significant change in cardiac function and has relatively poor perfusion, hypovolemia with associated oliguria and oligohydramnios[5]. If untreated, TTTS leads to perinatal death before 26 weeks in over 90% of diagnosed cases[4]. It is also associated with severe fetal morbidity and neurodevelopmental sequelae, preterm birth, growth restriction, cardiomyopathy, and hydrops fetalis. Fetoscopic laser coagulation (FLC) of the pathologic placental vascular anastomoses is the optimal fetal therapy, leading to survival of at least one fetus in 90% of pregnancies and minimizing both preterm birth and neurologic morbidity[5,6,7].

Early prediction of 'at risk' monochorionic twin pregnancies to aid the timely diagnosis of TTTS is clinically important, as early intervention, before there is significant cardiac dysfunction, may result in a more favorable overall prognosis[8] There has been much interest in the role of first trimester (11^{+0} - 13^{+6} weeks)

ultrasound biometric measurement of fetal crown rump length (CRL) and nuchal translucency (NT) in the prediction of adverse outcome in monochorionic twining. However, the individual measurements of CRL and NT and the intertwin differences have only a moderate predictive value for the development of TTTS, selective growth restriction (sGR) or indeed fetal demise; only 52% of cases deemed high risk ultimately develop TTTS[9]. This may be further refined by measurement of Ductus Venosus velocimetry[10] (as noted in small cohort studies) but overall the predictive value of first trimester ultrasound alone is disappointing. In the second trimester, the ultrasound detection of mild discordance in deepest vertical pool measurement in the amniotic sacs has a moderate ability to predict later onset of TTTS but again, as in the first trimester, sensitivity and specificity of prediction is not high[11,12]. Therefore, to improve early detection, novel approaches for screening are required using other modalities in addition to ultrasound.

Metabolomics is a powerful tool for investigating the final downstream products of genotype and environmental interactions and may be employed to investigate the complex interactions between specific metabolites and others as well as disease[13]. In a comparison of pre- and post-laser coagulation, Dunn and colleagues observed perturbations in carbohydrate and fatty acid levels in the fetal “recipient” amniotic fluid of monochorionic pregnancies complicated by TTTS[14], indicating that this pathological process may be associated with aberrant metabolism. Moreover, a profound change in the first-trimester metabolite profile of maternal plasma has been

noted in women who subsequently developed early-onset preeclampsia[15], indicating that metabolic changes in the first trimester may be an indicator of the occurrence or development of diseases.

In this 'pilot' study, **G**as **C**hromatography-**M**ass **S**pectrometry (GC-MS) was used in a metabolomics analysis of plasma collected at 11-15 weeks of gestation from pregnant women with monochorionic, diamniotic twin pregnancies (identified using ultrasound) that subsequently developed TTTS later in gestation. The finding from biochemical compounds and metabolic networks may enhance understanding of the maternal metabolic changes in early pregnancy associated with the occurrence of TTTS.

2. Methods

2.1. Patient recruitment

430 women with twin pregnancies were recruited in the Chongqing Longitudinal Twin Study (LoTiS)[16], which has been registered with the Chinese Clinical Trial Registry (ChiCTR-OOC-16008203). Of the 430 twin pregnancies, 117 were monochorionic diamniotic (MCDA), and 7 of the MCDA twin pregnancies developed TTTS (6%) (Figure 1). High-resolution fetal ultrasound was used to diagnose TTTS. The diagnosis was made using international criteria; if a monochorionic pregnancy had markedly discordant amniotic fluid volumes by a deepest vertical pool (DVP) >10 cm (100mm) at $>20^{+0}$ weeks' and >8 cm (80mm) at $<20^{+0}$ weeks' gestation in the recipient twin together with a DVP <2 cm (20mm) in the co-twin [17].

However, sGR was diagnosed when one twin had an estimated fetal weight of <10%[18]. As there are different diagnostic criteria for TTTS and sGR, sGR was excluded. Uncomplicated MCDA twin pregnancies were assigned to the non-TTTS group as controls; the gestation age was matched between TTTS and control groups when the plasma sample was collected in the first trimester (always in the morning with similar timing in the TTTS and control groups). The control group excluded MCDA twin pregnancies with the serious complications, of sGR, malformation and of courseTTTS (Figure 1). Participants were recruited at a median gestation of 13^{+1} (range 12^{+4} - 13^{+5}) weeks of gestation; five of the seven patients were diagnosed with Quintero stage I TTTS in the second trimester at a median gestation of 19^{+5} (19, 22^{+6}) week[19]. These MCDA twins where treated by fetoscopic laser coagulation and the outcome monitored until delivery (with overall 80% perinatal survival). A further two MCDA twin pregnancies were complicated by Stage III TTTS, diagnosed in the third trimester at 34^{+1} or 36^{+1} weeks and were delivered by cesarean section; all fetuses survived (100% survival).

2.2 Collection of samples

Maternal blood samples were collected into tubes with ethylene diamine tetra-acetic acid (EDTA) between 11-15 weeks (in the mornings) of gestation and then centrifuged at 4°C and 3000 rpm for 10 min. The plasma was stored at -80°C for future use.

208 **2.3 Sample preparation**

209 To extract the metabolites from plasma, 100 µl of plasma was added into the tubes
210 with 400 µl of pre-chilled methanol; an internal standard (20 µl of 2,3,3,3-d_4-alanine
211 (10 mM, Sigma-Aldrich, Missouri, USA)) was then added. The mixture was vortexed
212 for 1 min and incubated at -20°C for 30 min to precipitate protein. The supernatant
213 was collected after centrifugation (17,000 g, 15 min), dried by using a SpeedVac
214 (Labconco, Kansas, USA), and stored at -20°C for derivatization.

215

216 **2.4 Methyl chloroformate derivatization and GC-MS analysis**

217 Samples were chemically derivatized using a methyl chloroformate (MCF) method to
218 extract metabolites according to the protocol published by Smart *et al*[20]. Briefly, 200
219 µL of sodium hydroxide (1 M) was added to the dried samples, and 167 µL of
220 methanol and 34 µL of pyridine were added. Then, 20 µL of MCF was added with 30s
221 of vortexing, and the addition of another 20 µL of MCF was followed by 30s of
222 vortexing. Then, 400 µL of chloroform and 400 µL of sodium bicarbonate (50 mM)
223 were added and vortexed for 10s. The lower chloroform phase was used for GC-MS
224 analysis.

225

226 The samples were analyzed in a GC7890 system with an MSD5975 mass selective
227 detector (Agilent, California, USA). The MSD5975 mass selective detector (Agilent)
228 was a ZB-1701 GC capillary column (30 m x 250 µm id x 0.15 µm with 5-m guard
229 column, Phenomenex, California, USA). One microliter of sample was injected into

the GC inlet. Helium gas was the carrier gas flow at 1 mL/min. The auxiliary temperature, MS quadrupole, and MS source were 250°C, 230°C, and 150°C, respectively, and the scan speed was 1.562 u/s.

2.5 Data analysis and statistics

Automated Mass Spectral Deconvolution & Identification System (AMDIS) software was used to de-convolute GC-MS chromatograms and identify metabolites with our in-house MCF. The metabolite values were normalized to the abundance of the internal standard and total ion count. Significant metabolites were analyzed with the limma R package. Principal component analysis (PCA) was based on data from all metabolites to visualize the differences between TTTS and control group. Log transformation was used to make the distributions of the data more similar to a Gaussian distribution. Mann–Whitney U-test, Chi square test, Student's t-test and box plot were used in R and SPSS 24.0. The discrepancy in the CRL of the twins was calculated by subtracting the CRL of the smaller fetus from that of the larger one and expressing this as a quotient of the larger fetus CRL[21]. Power analysis was applied in website (https://www.metaboanalyst.ca/). PAPi enrichment approach was applied to compare metabolic pathways activities based on R-software package, then t-test was used to investigate difference between TTTS and control group [22]. The metabolic pathways with p-values less than 0.05 and q-values less than 0.30 were considered statistically significant. Based on KEGG database, Cytoscape [23]was used to visualize and interpret metabolomic data in the context of metabolic networks.

3. Results

Population characteristics

Seven TTTS pregnancies and seven control MCDA pregnancies from LoTiS were included in this pilot study. A retrospective calculation of a sample size of 7 cases and 7 controls available in this study, indicated we had a power of 0.67 to detect a difference of 1.2 folds in metabolite abundance at a false discovery rate of 5% (supplementary Figure 1). The gestational age at diagnosis of the five second trimester TTTS patients was 19^{+5} weeks (19, 22^{+6}). The other two patients were diagnosed with late-onset acute TTTS stage III at 34^{+1} and 36^{+6} weeks', respectively. Clinical maternal and fetal characteristics are outlined in Table 1. There were no significant differences in maternal age, nuchal translucency (NT), crown–rump length (CRL) discrepancy, gestational age and fetal gender, between the two groups at sample collection. Maternal pre-pregnancy BMI, CRL, gestational age at delivery (32^{+2}-35^{+3}, 37^{+2} -37^{+3} weeks'), larger and smaller amniotic fluid volume, amniotic fluid volume difference, and birth weight of the large fetus were significantly different between TTTS and control groups.

Metabolome of maternal plasma in the control versus TTTS group

In the maternal plasma samples that were collected between the 11^{th} and 15^{th} weeks of gestation, there were over 200 individual spectral peaks separated by gas chromatography. PCA analysis showed that the metabolites were notably separated (Figure 2). Significance is assessed via moderated t-tests implemented in the limma

package of Bioconductor, 17 metabolites were significantly differed in plasma from the control and TTTS groups (Figure 3). Specifically, the levels of saturated fatty acids (palmitic acid, pentadecanoic acid and myristic acid), unsaturated fatty acids (9-heptadecenoic acid and linolelaidic acid), organic acids (4-aminobutyric acid, D-fumaric acid, 2-methyloctadecanoic acid and cabamic acid), TCA cycle and intermediate acids (oxaloacetic acid), alkanes (pentadecane), nicotinamide, an amino acid derivative (beta-alanine) and cysteine were decreased in the TTTS group compared to the control group, while the levels of amino acid (methionine and glycine) were increased in the TTTS group.

Disrupted metabolic pathways in the maternal plasma of TTTS pregnancies from 11-16 weeks of gestation

To elucidate the metabolic pathways that were different in pregnancies complicated by TTTS, all of the identified metabolites were subjected to pathway analysis, and the data suggested that 13 metabolic pathways were significantly altered in TTTS-complicated pregnancies from 11-15 weeks of gestation (Figure 4a): amino acid metabolism, carbohydrate metabolism, cofactors, vitamins, and purine were downregulated, and bile secretion and pyrimidine metabolism were upregulated. A metabolic network was constructed based on these results (Figure 4b). Intriguingly, glycine and cysteine were the nexus of the network. The correlation between identified metabolites and metabolic pathways is illustrated in Figure 4c, in which the involved pathway is predicted. Taken together, these findings suggest that

perturbations in metabolic pathways of maternal blood during early gestation may be associated with subsequent TTTS development.

Discussion

Pregnancy is characterized by its complex metabolic processes that can impact fetal development[24]. Given the potential high fetal mortality of TTTS without appropriate fetal therapy, clinical evidence suggests that the optimal time frame for fetoscopy in TTTS is 16-26 weeks of gestation.[6] Predictive metabolic biomarkers (before 16 weeks) may aid timely identification of "at risk" monochorionic twin pregnancies and a potentially diagnosis of TTTS at a lower Quintero stage with improved double survival rates[8]. This 'proof of principle study' noted metabolic perturbations in maternal plasma at 11-15 weeks of gestation in apparently uncomplicated monochorionic, diamniotic twin pregnancies prior to the subsequent ultrasound diagnosis of TTTS.

Dunn and colleagues have previously reported higher levels of fatty acids and lower levels of carbohydrates in amniotic fluid of post-FLC; they suggested that metabolic disorders existed in TTTS, and the changes were associated with fetal or placental energy metabolism and the echocardiographic measures of recipient cardiac function[14].

A role for fatty acids in the pathogenesis of TTTS is biologically plausible. Fatty acids can be imported, oxidized, synthesized, and exported by endothelial cells, and fatty

acid oxidation contributes to approximately 5% of total cellular ATP production in endothelial cells[25]. Oxidation of fatty acids is irreplaceable for endothelial cell proliferation because carbons are provided for de novo nucleotide synthesis[26]. Hence, fatty acids are a metabolic determinant of the angiogenic process. Many studies have shown that fatty acids are key to pregnancy outcomes. Lipid metabolic genes are upregulated in the first trimester, and fatty acid uptake by placental trophoblast cells is associated with angiogenic processes[27]. Fatty acids are not only a substantial energy source but also have effects on membrane fluidity, intracellular cell-signaling cascades and susceptibility to oxidative injury[28]. Linolelaidic acid has been shown to exhibit a proliferative effect on human umbilical vein smooth muscle cells[29]. Trophoblasts oxidize palmitate and myristate for energy[30]. During normal pregnancy, vascular remodeling, fatty acid synthesis and transport, and lipid droplet formation are increased[31]. Fatty acids are key to angiogenesis and thus play an important role in human placentation[32,33]. Maternal angiogenic activity is decreased in severe TTTS [34]. However, in our study, we found that the decrease of saturated fatty acids and unsaturated fatty acids in TTTS group, which indicated that the utilization of fatty acids in TTTS pregnant women was increased. Increased oxidative utilization of fatty acids can provide energy for vascular remodeling of placenta. Fatty acids are important components of cell membrane. Therefore, increased utilization of fatty acids in the first trimester can promote the process of pathological placental vascular anastomosis in TTTS patients, and it may develop into an early metabolic disorder that disrupts placental angiogenesis and the fetal energy supplies.

Recently, amino acid metabolism was found to play important roles in regulating and maintaining various aspects of vascular function, such as vascular tone, coagulation and fibrinolysis, cell growth and differentiation, redox homeostasis, and immune and inflammatory responses[35]. In this study, we found that methionine and glycine were upregulated in the maternal plasma of pregnancies subsequently complicated by TTTS and that these metabolites positively correlated with abnormal amniotic fluid depth. Alterations in amino acid levels between donor and recipient twins in TTTS and between non-TTTS twin pairs have previously been reported, due to intertwin transfusion that impairs placental transport of amino acids[36]. In placental insufficiency, maternal cysteine and methionine levels are decreased, and maternal glycine is increased[37]. Amino acids enhance protein synthesis and cell proliferation in the placenta[38]. Glycine can promote angiogenesis[39]. Methionine, a sulfur-containing amino acid, is a major component in protein synthesis[35], and blockade of methionine metabolism in endothelial cells leads to endothelial damage or inflammation[40]. In the metabolome of amniotic fluid, higher levels of carbohydrates in TTTS pregnancies before fetoscopic laser coagulation treatment and higher levels of fatty acids post-treatment[14], indicate that this treatment can partially correct metabolic changes. These studies suggest that amino acids are associated with placentation and angiogenesis. In addition, upregulated methionine and glycine levels may be a sign that indicates early changes in the metabolic microenvironment during the first

trimester in women whose pregnancies are subsequently complicated by TTTS.

Because of the low incidence of TTTS in monochorionic twin pregnancies (~10%), it is difficult to investigate the manifestations of TTTS risk in early pregnancy maternal blood. A prospective study in a large cohort of monochorionic twins pregnancies would be required and the improved identification of ‘at risk’ pregnancies when metabolic profiles are used alone or in combination with fetal ultrasound variable would be desirable[41]. The aim of this work was to evaluate the prognostic ability of individual first-trimester metabolomic biomarkers for TTTS in MC twins. For those metabolomic biomarkers where there was a statistically significant independent association with adverse outcome, calculating the absolute risk for each outcome using common values demonstrated that the markers may be useful clinically, although this would depends on a change in management consequent on the risk assessment[42].

Limitations

As the lower statistic power, we should expand a significantly larger prospective study of monochorionic twins to enable the development of a prognostic model. Secondly, another limit is that we only tested metabolites of maternal blood, rather the blood metabolome not likely reflects disrupted metabolism in unspecified uterus and fetal development. Thirdly, our study was not a randomized controlled study, but the results also could provide scientific support for future large randomized controlled cohort studies. Fourthly, early diagnosis is of great significance for the detection of

clinical diseases, but we cannot exclude that monochorionic twins will not develop to TTTS just because there is no difference in metabonomic of blood samples in the first trimester of pregnancy. The next step of our future work is to replicate the study in independent samples. Finally, the study should be performed and analyzed according to robust prognostic methodology, which has been reported by McShane and colleagues for predictive markers studies[43].

Conclusions

Although the idea that TTTS can potentially be detected using this methodology is exciting, to our knowledge, this is the first report of a metabolomic profile with human plasma for predicting the occurrence of TTTS before 16 weeks. This study provides insights into the relationship between early metabolic changes and the occurrence of TTTS. Based on our results, we found that amino acid metabolism, carbohydrate metabolism and lipid metabolism were altered and that the abnormal changes may be related to subsequent placental development and angiogenesis.

Authors' contributions

C.T, J.L and H.Q conceived the study, which was discussed with RS, MDK and PB at regular Chongqing 111 study meetings; Y.Y, L.W, T.H performed experiments and analyzed the data; L.Z, H.F and J.G collected samples and clinical data; Y.Y and C.T drafted the manuscript; R.S, M.D.K and P.B commented on the data and revised the manuscript.

406

407 **Ethics approval and consent to participate**

408 This study was approved by The Ethics Committee of The First Affiliate Hospital of
409 Chongqing Medical University (approval number: 201530; date of approval:
410 2/29/2016). And each participant will sign the informed consent before
411 re-investigation.

412

413 **Consent for publication**: Not applicable.

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Table 1. Clinical characteristics of subjects.

583 **Figure legend**

584 **Figure 1. Flowchart showing selection of TTTS and control participants**

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Figure 2. Principal component analysis (PCA) of TTTS and controls.

PC1 vs. PC2. Score plot shows separation between TTTS cases (green) and controls (red) based on significant differences in metabolite abundance ($p<0.01$) between the groups (n=7 in each group).

Figure 3. Altered metabolites in the maternal plasma of TTTS versus control pregnancies between 12 and 15 weeks of gestation.

The boxplots show the significant metabolites with their relative concentrations. Blue boxes indicate TTTS group, red boxes indicate Control group. Only the metabolites with p-values less than 0.05 and q-values less than 0.12 are displayed in the figure.

Figure 4. Predicted alterations in metabolic pathways in TTTS between 11 and 16 weeks of gestation.

A. Predicted metabolic changes in TTTS compared to normal controls; red represents increased metabolism, whereas green represents downregulated metabolism. Only the metabolic pathways with p-values and q-values less than 0.05 are displayed in the heatmap. B. The metabolic network plotted according to the metabolites identified in maternal plasma collected at 12-15 weeks of gestational age is shown. The red dots represent metabolites that were significantly upregulated in TTTS, while the blue dots represent metabolites that were significantly downregulated in TTTS. The yellow dots represent the metabolites in plasma that showed no differences between TTTS and normal controls. The smaller yellow circles are unidentified metabolites that were directly connected to the identified metabolites. C. Circles indicate the connections between metabolic pathways and metabolites.