

Selective Intrauterine Growth Restriction in Monochorionic Twin Pregnancies: Markers of Endothelial Damage and Metabolomic Profile

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The aim of this study was to assess the aorta–intima thickness (aIT) and serum metabolomic profile in selective intrauterine growth-restricted (sIUGR) monochorionic diamniotic (MCDA) twin fetuses presenting Doppler velocimetry alterations. Fetal abdominal aIT was measured by ultrasound at 32 weeks of gestation, enrolling 24 MCDA twin fetuses (8 sIUGR and 16 controls). sIUGR twin fetuses were classified into two groups: Group 1 consisted of sIUGR with abnormal umbilical artery (UA) Doppler waveforms and Group 2 included sIUGR with normal UA Doppler. Group 3 were control fetuses appropriate for gestational age (AGA). Fetal blood samples were obtained from the umbilical vein immediately after fetal extraction. A non-targeted metabolomic profiling investigated fetal metabolism alterations by using liquid chromatography–high-resolution mass spectrometry (LC–HRMS). Median fetal aIT was significantly larger in Group 1 (median value = 0.9 mm; range = 0.8–1.0 mm; $p < .002$) and Group 2 (median value = 0.8 mm; range = 0.7–0.8 mm; $p < .002$) than in AGA Group 3 (median value = 0.5 mm; range = 0.4–0.6 mm; $p < .002$). Metabolomic analyses, performed on four sIUGR cases (Group 1) compared with four AGA co-twins, showed an upregulation of phenylalanine, sphingosine, glycerophosphocholine, and choline, and a downregulation of valine, tryptophan, isoleucine, and proline sIUGR Group 1 compared with AGA. Although for metabolomics data only a statistical tendency (and not a statistical significance) was reached due to the small sample size, we believe that our results represent a valid starting point for further in-depth metabolomic and proteomic investigations of sIUGR in MCDA fetuses.

■ **Keywords:** twin pregnancies, growth restriction, IUGR, endothelial damage, metabolomics

Thought to represent a special category of stunted fetal growth, twins have a higher risk of diseases associated with lower birth weight and major incidence of morbidity and mortality (Blickstein & Kalish, 2003). In particular, monochorionicity has been associated with an increased rate of adverse perinatal outcomes when compared with dichorionicity. This is in part due to a higher incidence of intrauterine growth restriction (IUGR), abnormal placental vascular anastomoses, fetal anomalies, and stillbirth. Compared with singletons, the growth rate of twin fetuses is often characterized by a higher incidence of IUGR in one of the fetuses, varying from 15% to 25% in all monochorionic (MC) pregnancies, according to the definition of the American College of Obstetricians and Gynecologists (ACOG), and frequently associated with Doppler velocimetry alterations (Amaru et al., 2004; Committee on Practice Bulletins — Gynecology, 2001; Gratacos et al., 2004).

It is now well known that IUGR is a condition associated with an increased risk of adult onset disease. The fetal origin hypothesis in fact proposes that cardiovascular diseases and type 2 diabetes mellitus in adult life are associated with low birth weight, through several adaptation mechanisms of the undernourished fetus in the structure and function of the body (Barker, 2000, 2006; Irving et al., 2000; Osmond & Barker, 2000).

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Early endothelial dysfunction and aortic wall intima-media thickening (aIMT) occurring in utero may play an important role in premature in utero stiffening of the aortic vessels (Skilton et al., 2005). Previous studies have demonstrated an association between low birth weight and abdominal aorta intima-media thickness in utero and at 18-month follow-up, and high blood pressure, and microalbuminuria in childhood (Cosmi et al., 2009; Koklu et al., 2007; Zanardo et al., 2011).

Twins present a distribution of birth weights shifted to the left of the normal singleton distribution; it would be expected that twins may be at increased risk for the long-term outcomes, but the relationship between birth weight and the onset of adult diseases in those born as a twin is unclear, with only scarce evidence in the literature (Joseph et al., 2003; Lopriore et al., 2012; McMillen & Robinson, 2005). In this perspective, research on twin fetuses provides a unique opportunity to mimic a scientific experiment to study IUGR and, reflecting nutritional stresses within a similar genetic fetal background, to distinguish between genetic and environmental causes of phenotypic variations in the human population (Muhlhusler et al., 2011). The cause of discordant growth in MC twin pregnancies probably originates from a variety of conditions: limited maternal uterine environment, intertwin distribution of blood and nutrients along vascular placental anastomosis, unequal placental sharing, abnormal cord insertion, or impaired transport of amino acids through the placenta (Bajoria et al., 2001; Ijzerman et al., 2005; Ropacka-Lesiak et al., 2012; Valsky et al., 2010).

Metabolomics, a relatively new approach used for characterizing the metabolic phenotype of individuals by integrating genetic, epigenetic, and post-transcriptional data, has recently been applied in the field of nutritional fetal and pediatric research (Alexandre-Gouabau et al., 2011; Dessì et al., 2012). Utilizing this technique, our previous studies detected significant differences in the distribution of essential amino acids (Favretto et al., 2012) and in the expression of proteins (Cecconi et al., 2011) between IUGR and appropriate for gestational age (AGA) single fetuses.

The aim of this study was (1) to compare, in a selected sample, the abdominal aorta-intima thickness (aIT) among selective intrauterine growth restricted (sIUGR) twin fetuses with and without umbilical artery (UA) Doppler vasculopathy and AGA twin fetuses in utero; (2) to compare metabolomic profiles of MC sIUGR twins with UA Doppler abnormalities to controls; and (3) to test the hypothesis of an association between aIT and metabolic profiles in IUGR twins with UA Doppler alterations.

Materials and Methods

Patients

This study was carried out in 24 monochorionic diamniotic (MCDA) twins from 12 pregnancies with and without

IUGR at the Center for Prenatal Diagnosis of the University of Padova. The institutional review board and the ethics committee for clinical studies of the University Hospital approved the study protocol, and all patients or their guardians provided informed consent. Patients were selected from a larger cohort included in a recently published study on monochorionic and dichorionic twin pregnancies (Visentin et al., 2013).

The diagnosis of monochorionicity was performed at 10–14 weeks of gestation in the presence of a single placental mass and the ‘T’ sign (Carroll et al., 2002), and was confirmed at birth by histology. Gestational age was calculated on the basis of the first day of the last menstrual period and confirmed by the ultrasound measurement of the fetal crown-rump length (CRL; Drumm, 1977).

sIUGR in MCDA pregnancies was defined as an estimated fetal weight (EFW) below the 10th percentile in the smaller twin and above the 10th percentile in the larger one (Ananth et al., 1998; Fox et al., 2011). sIUGR twins were further divided into two groups based on UA Doppler waveforms: Group 1 included sIUGR twins with abnormal UA Doppler (UA Pulsatility Index [PI] > 2 standard deviation [SD], absent or reverse end-diastolic flow); Group 2 included sIUGR twins with normal UA Doppler and with normal end-diastolic flow. MC twins with an EFW above the 10th percentile, confirmed after birth, and normal UA Doppler constituted the AGA control group (Group 3). Table 1 presents a summary of the studied cases.

Exclusion criteria were as follows: unknown last menstrual period and chorionicity; triplet pregnancy; twin-to-twin transfusion syndrome (TTTS) or related conditions; MC monoamniotic twin pregnancies; first trimester discrepancy of CRL among the pair of twins > 5 days; structural or chromosomal abnormalities; single intrauterine death or selective feticide; maternal history of cardiovascular disease or endocrine disorders such as diabetes, hypercholesterolemia, pre-eclampsia, thyroid and adrenal problems; clinical chorioamnionitis; as well as maternal consumption of alcohol, drugs of abuse or nicotine. TTTS was defined according to the Quintero classification (Quintero et al., 1999).

aIT and diameter measurements were determined for each twin at a median gestational age of 32 weeks (range 28–36 weeks) in a coronal or sagittal view of the fetus at the arterial wall of the most distal 15 mm of the abdominal aorta, sampled below the renal and above the iliac arteries. Abdominal aIT was measured placing the calipers at the leading edge of the blood-intima interface and at the end of the inner portion of the intima vessel. We chose this measurement instead of aIMT because a recent study of our group on fetal aorta sampled after intrauterine fetal demise showed an increase of the intima of the aorta in the IUGR stillbirth (Lo Vasco et al., 2011). All images were stored digitally for off-line analysis. All measurements were performed in high resolution with the same ultrasound

scan with a 3.5- to 5-MHz linear array transducer (Antares, Siemens Medical Solutions, Mountain View, CA).

Three aIT measurements were taken and the median was considered. All images were captured during the last phase of the cardiac cycle to minimize variability. The aortic diameter was measured at the same level of aIT, from the inner wall to the wall edges. End diastole was determined as the maximal expansion of the vessel using the cine loop capability of the ultrasound machine once the images of the entire cardiac cycle were frozen. Each measurement was taken during fetal apnea after three consecutive, similar waveforms were obtained. Pulsatility index and time-averaged velocities (defined as the area under the velocity spectral envelope) were measured using the machine software.

Before starting the main research, the intraobserver and interobserver agreements were evaluated in the measurement of aIT, as reported elsewhere (Visentin et al., 2013).

All MC pregnancies were also monitored by routine ultrasound examination, including a complete morphological examination, estimation of fetal weight, amniotic fluid index calculations, and Doppler assessment of the UA, middle cerebral artery (MCA), and ductus venosus (DV).

Sample Collection

Fetal blood samples (0.5 mL) were obtained from the umbilical vein from a doubly clamped segment of each cord immediately after fetal extraction, and were collected in a Vacutainer SST II Advance (BP Diagnostic, Belliver Industrial Estate, Plymouth, UK) tube and centrifuged at $1,500 \times g$ for 10 minutes at 4 °C. In none of the cases did the time from birth to sample collection exceed 10 minutes. Time was recorded with a stopwatch. Additional umbilical arterial and venous samples from a segment of cord that was clamped at both ends were also obtained for the determination of acid–base status as routine clinical practice.

The samples were collected at the Department of Health of the Woman and Child and were analyzed at the Department of Molecular Medicine, Forensic Toxicology and Antidoping Unit, University of Padova.

Sample Preparation

Serum samples (200 μ L) were deproteinized by mixing with methanol (600 μ L) at room temperature, vortex mixed for 20 seconds, and incubated at -20 °C overnight. Samples were then centrifuged ($13,845 \times g$, 15 minutes, 4 °C). Supernatants were transferred to Eppendorf tubes, lyophilized, and kept at 4 °C. Samples were reconstituted in 100 μ L of water prior to analysis.

Chemicals

Twenty essential amino acids (Gly, Ala, Ser, Pro, Val, Thr, Cys, Leu, Ile, Asn, Asp, Gln, Lys, Glu, Met, His, Phe, Arg, Tyr, and Trp) were purchased from Sigma (Saint Quentin

Fallavier, France). Standards were dissolved in water to obtain solutions of 50 μ mol/L before liquid chromatography (LC) tandem mass spectrometry analysis (MS/MS).

The standard mixtures used for the external calibration of the MS instrument (caffeine, L-Ultramark 1621, sodium dodecyl sulfate, and sodium taurocholate) were from Thermo Fisher Scientific (Les Ulis, France). Methanol, formic acid, and acetonitrile were from Merck (Briare-lecanal, France).

All the chemicals used were of analytical or reagent grade.

Metabolomic LC-HRMS Analysis

Liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analysis was obtained using an LTQ-Orbitrap mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany) connected to a Surveyor Plus LC. High performance LC separation was performed with a Luna C18 (1.0 \times 150-mm) column (Phenomenex, Torrance, CA) with a constant flow rate of 100 μ L/min. The LC eluents were water, 0.1% formic acid (phase A) and methanol, and 0.1% formic acid (phase B). A chromatographic gradient was used. After an isocratic step of 1 minute at 90% phase A and 10% phase B, a linear gradient from 10% to 100% B was run over the next 35 minutes, with a mobile phase flow of 100 μ L/min. A 10- μ L sample volume was injected into the column. An electrospray source was used, working in positive ion mode. For the positive ion detection mode, the sheath gas was set at 13 (arbitrary units) and the auxiliary gas at 29 (arbitrary units), and capillary temperature was 275 °C. The spray voltage and tube lens voltage were 4,500 and 85 V, respectively. Lens parameters were optimized with a tune solution containing 14 metabolites (cystine, arginine hydrochloride, arginine, tryptophan, lysine monohydrochloride, lysine, tyrosine, serine, valine, leucine, asparagine, glutamic acid, methionine, and histidine) at a concentration of 1–3 mmol/L and infused at 5 μ L/min. Gas flows were then optimized with the same compounds but with a 0.1-mL/min LC flow.

Orbitrap calibration was performed in the positive ion mode. Full-scan mass spectra were acquired over the m/z range of 50–1,000 at a speed of 0.45 s/scan (2 μ scans) using the Orbitrap mass analyzer operating with a target mass resolving power of approximately 30,000 (full-width at half-maximum of peak).

To account for any analytical variability, measurements were performed in triplicate (three injections from the same sample).

All data were processed with Qualbrowser (Thermo Fisher) and its chemical formula generator was used to provide elemental compositions with a relative error lower than 5 ppm.

Raw data from analysis were transformed to peak tables by the XCMS software (<http://massspec.scripps.edu/xcms/xcms.php>), including statistical tools (see below).

Metabolomic Data Analysis and Statistics

Data preprocessing. Raw LC-HRMS data were pre-processed for untargeted metabolite profiling with the open-source package XCMS (Smith et al., 2006), written in R statistical programming language, available at Metlin Metabolite Database and the Bioconductor Bioinformatics Project (Gentleman et al., 2004). Peak matching and single-step retention time alignment of all samples were obtained automatically, and a matrix of aligned peaks was produced in .tsv format. Values close to zero due to baseline noise were filtered and excluded, since these values can interfere with computational algorithms in further data analysis. Peak-matching algorithm considered a bandwidth (*SD* or half-width at half-maximum) of 30 for the Gaussian smoothing kernel and fixed-interval bins 0.25 *m/z* wide. Retention time alignment algorithm was the Loess method with an automated outlier detection and removal. The abundance of each ionic species was normalized according to the total ion current (TIC) measured for each sample and expressed as percentage.

Statistics. To identify the most relevant differences between groups, a univariate statistical analysis was used on spectral data, considering each neonate as an independent unit. Filtering procedures, such as fold-change analysis and *t* tests (for paired and unpaired data) were applied, as provided by MetaboAnalyst web-based software (Xia et al., 2009; Xia & Wishart, 2011). Significantly different data at the probability level of $p < .05$ were used for further procedures of the multivariate analysis, to obtain the identification of relevant biomarkers. One-way ANOVA followed by post hoc tests was used to detect differences among groups. Differences were judged statistically significant at $p < .05$. Tentatively, the entire metabolomic dataset was submitted to the multivariate analysis (with MetaboAnalyst; Xia et al., 2009; Xia & Wishart, 2011). The procedure is explained in the supplementary material.

Metabolite identification. The putative masses responsible for the metabolic differentiation among the three groups were used to make queries in the METLIN (<http://metlin.scripps.edu/>), Mass Bank Database (<http://www.massbank.jp/en/database.html>), and Human Metabolome (<http://www.hmdb.ca/>) online databases in order to obtain the corresponding chemical structures. The identities of metabolites of particular interest were confirmed by a comparison of their retention times, accurate mass spectra, and HRMS information with those of authentic chemical standards. The KEGGs Database (<http://www.genome.jp/kegg/>) was used to identify metabolic pathways of interest.

Results

The study was conducted on a total of 12 consecutive MCDA twin pairs: four sIUGR neonates in Group 1, four sIUGR

TABLE 1
Characteristics of the Cases

	Sample ID	Type
Pregnancy 1	1A	Group 3
	1B	Group 3
Pregnancy 2	2A	Group 3
	2B	Group 1
Pregnancy 3	3A	Group 3
	3B	Group 3
Pregnancy 4	4A	Group 3
	4B	Group 3
Pregnancy 5	5A	Group 3
	5B	Group 1
Pregnancy 6	6A	Group 1
	6B	Group 3
Pregnancy 7	7A	Group 3
	7B	Group 2
Pregnancy 8	8A	Group 2
	8B	Group 3
Pregnancy 9	9A	Group 2
	9B	Group 3
Pregnancy 10	10A	Group 3
	10B	Group 2
Pregnancy 11	11A	Group 3
	11B	Group 1
Pregnancy 12	12A	Group 3
	12B	Group 3

Note: Bold type indicates the pairs of twins (each one independently considered) that were analyzed by a multivariate approach (see Results section). Group 1 = sIUGR with abnormal UA Doppler waveforms; Group 2 = sIUGR with normal UA Doppler waveforms; Group 3 = AGA twins.

neonates in Group 2, and 16 AGA in Group 3. For eight neonates, the pair consisted of an sIUGR twin (four with UA Doppler alterations and four without) and the corresponding AGA co-twin (Table 1).

Maternal and neonatal data are shown in Table 2. There were no differences in maternal age and ethnicity among the groups. All the patients were subjected to cesarean section for fetal reasons or in a planned way as per the internal protocol of the hospital. Birth weight and length were significantly diminished in the sIUGR Group 1, as well as pH of cord blood. Instead, there were no differences among the groups in Apgar score 5 minutes after birth and in base excess.

Both Groups 1 and 2 twins showed an aIT value significantly greater than that of Group 3, but the difference was not significant when comparing Groups 1 and 2. As previously demonstrated, aIT correlated with Doppler UA abnormalities (Cosmi et al., 2009; Visentin et al., 2013).

The metabolomic analysis was carried out comparing the LC-HRMS data obtained from the three different groups of twin pregnancies, according to their status. In a first set of multivariate statistical analysis, the three groups of twins were compared; PCA analysis and cluster analysis (Ward's method) did not allow a clear distinction between the three groups; in some cases the pattern of ionic species identified appeared consistent among paired twins, but not always belonging to same pathology classes (Figures S1–S2 in the supplementary material).

TABLE 2
Maternal and Neonatal Data

Parameters	Group 1	Group 2	Group 3	Overall <i>p</i> value
Twins (no.)	4	4	16	
Maternal age (years)	31 (24–37)	33 (32–35)	29 (24–37)	.26
Primiparous (%)	50%	100%	75%	.18
aIT (mm)	0.9 (0.8–1.0)**	0.8 (0.7–0.9)**	0.5 (0.4–0.6)	.0002
Aortic diameter (mm)	3.5 (2–4.1)	3.2 (2.7–4)	4.1 (2.9–4.9)	.20
Umbilical artery Doppler waveforms (no.)				
Normal	0**	4†	16	.0002
PI > 2 SD	4**	0†	0	.0002
AEDF/ARED UA	3*	0	0	.004
Presence of A-wave in DV	4	4	16	1
Mode of delivery (no.)				
Cesarean	4	4	16	1
Vaginal	0	0	0	1
Gestational age at delivery (wk)	33 (28–36)	33 (33–36)	35 (28–36)	.47
Birth weight (g)	1,480 (870–1,725)*	1,970 (1,680–2,260)	2,315 (1,210–3,030)	.01
Birth height (cm)	39.5 (35–41)*	43 (42–45)†	45 (37–47)	.006
Apgar at 5 minutes	9 (8–9)	9 (9–10)	9 (6–10)	.42
pH cord blood at delivery	7.1 (7.1–7.2)*	7.2 (7.2–7.3)	7.3 (7.2–7.3)	.004
BE cord blood	3.7 (1.6–6.2)	4.7 (3.2–8.5)	4.3 (1.6–6.7)	.54

Note: Values for continuous variables are given as median and range. Group 1 = sIUGR with abnormal UA Doppler waveforms; Group 2 = sIUGR with normal UA Doppler waveforms; Group 3 = AGA twins.

aIT = aorta-intima thickness; PI = Pulsatility Index; AEDF/ARED = absent and reverse diastolic flow; UA = umbilical artery; SD = standard deviation; DV = ductus venosus; BE = base excess.

For continuous variables, difference among groups was evaluated by using the Kruskal–Wallis test followed by a non-parametric Wilcoxon test to assess the difference between pairs. For frequency data, difference was evaluated with the Fisher exact probability test.

***p* < .002; **p* < .01, comparisons versus Group 3; †*p* < .05, comparison Group 1 versus Group 2.

Another approach was then tested: only pairs of twins of which one was normal and the other affected by sIUGR with abnormal UA Doppler waveforms were considered (i.e., four sIUGR vs. four AGA neonates). Due to the rarity of this condition, only four paired cases were available (cases from Groups 1 and 3 shown in bold type in Table 1).

In considering the four pairs of twins, a multivariate approach was tried first with paired test option (MetaboAnalyst; Xia et al., 2009; Xia & Wishart, 2011). The most relevant ionic species that resulted from a preliminary multivariate analysis were selected, and a further PCA and cluster analysis were performed. Although PCA did not allow a satisfactory separation of the groups (Figure S3 in the supplementary material), an appreciable clustering of data was obtained following cluster analysis (Figure S4). The multivariate approach permitted the identification of the most relevant variations by using the fold-change value between the two groups, controls (Group 3, AGA) and sIUGR (Group 1). Figure 1 indicates the ionic species that were the most different between the pairs of AGA (Group 3) and sIUGR (Group 1) co-twins. The low sample size and the large dispersion of data did not reach a statistically significant difference (Student's *t* test for paired data did not reveal a significant difference between the AGA Group 3/sIUGR Group 1); however, a peculiar trend was observed, with some ionic species being more abundant in AGA (positive δ) and others more abundant in sIUGR Group 1 twins (negative δ).

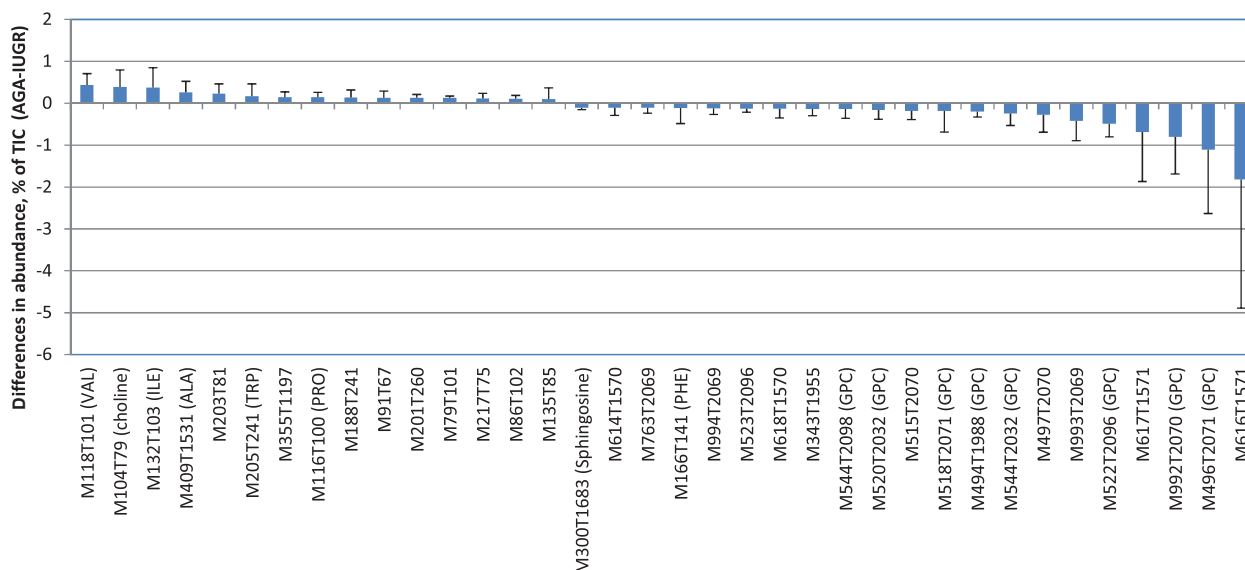
A search using the Metlin database identified several of these ionic species (Table 3). As shown in Table 3 and Fig-

TABLE 3
Ionic Species Identified in the LC-HRMS Spectra

Metabolites	Elemental composition	Label, according to XCMS output
Valine	C ₅ H ₁₁ NO ₂	M118T101
Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	M205T241
Proline	C ₅ H ₉ NO ₂	M116T100
Isoleucine	C ₆ H ₁₃ NO ₂	M132T103
Phenylalanine	C ₉ H ₁₁ NO ₂	M166T141
Choline	C ₅ H ₁₃ NO	M104T79
Glycerophosphocholine	C ₂₆ H ₅₂ NO ₇ P	M522T2096
		M544T2098
		M520T2032
		M518T2071
		M544T2032
		M496T2071
Sphingosine	C ₁₈ H ₃₇ NO ₂	M300T1683

ure 1, some of the species identified were amino acids. Concentrations of the essential amino acids, valine and isoleucine, and non-essential amino acids, tryptophan and proline, were lower in Group 1 than in AGA co-twins; on the contrary, phenylalanine appeared upregulated in sIUGR Group 1 twins compared with AGA ones.

Figure 2 presents a comparison of cumulative absolute abundance (in arbitrary units; a.u.) of total ionic currents (TIC) for the amino acids in the four pairs of twins. As outlined in Figure 2(a), when the sum of TIC for the amino acids valine, tryptophan, proline, isoleucine is plotted, the control twins always show an upregulation. On the contrary, as shown in Figure 2(b), when the sum of TIC for the amino acid phenylalanine is plotted, the trend is reversed,


FIGURE 1

(Colour online) Species presenting major changes between the respective AGA neonates (Group 3) and sIUGR neonates with abnormal UA Doppler waveforms (Group 1); data are the mean difference (\pm SEM) in the abundance of the metabolites determined in four twin pairs, of which one baby was normal (AGA) and the other affected by sIUGR with abnormal UA Doppler waveforms (Group 1). The identified species are indicated in the labels (Table 3). GPC = glycerophosphocholine.

with Group 1 twins overexpressing this aromatic, essential amino acid. In this case, however, the overexpression is not markedly evident.

Figure 3 reports the comparison of absolute abundance (a.a.) of TIC for the other ionic species identified: choline (Figure 3a), sphingosine (Figure 3b), and the different isomers of glycerophosphocholine (Figure 3c); choline is down-regulated in sIUGR Group 1 twins, whereas the other species are upregulated.

Discussion

To the best of our knowledge, this study is the first to investigate endothelial structure in a selected group of MCDA twins, measuring the abdominal fetal aIT and performing metabolomic analyses on cord blood collected immediately after birth. The data collected evidenced a higher aIT in sIUGR with abnormal UA Doppler waveforms, with respect to AGA. Additionally, the metabolomic investigation showed several differences in the concentration of essential and non-essential amino acids in sIUGR neonates (four belonging to Group 1) compared with their MCDA co-twins (four AGA neonates), although, due to the small number of cases, the measured differences did not reach statistical significance.

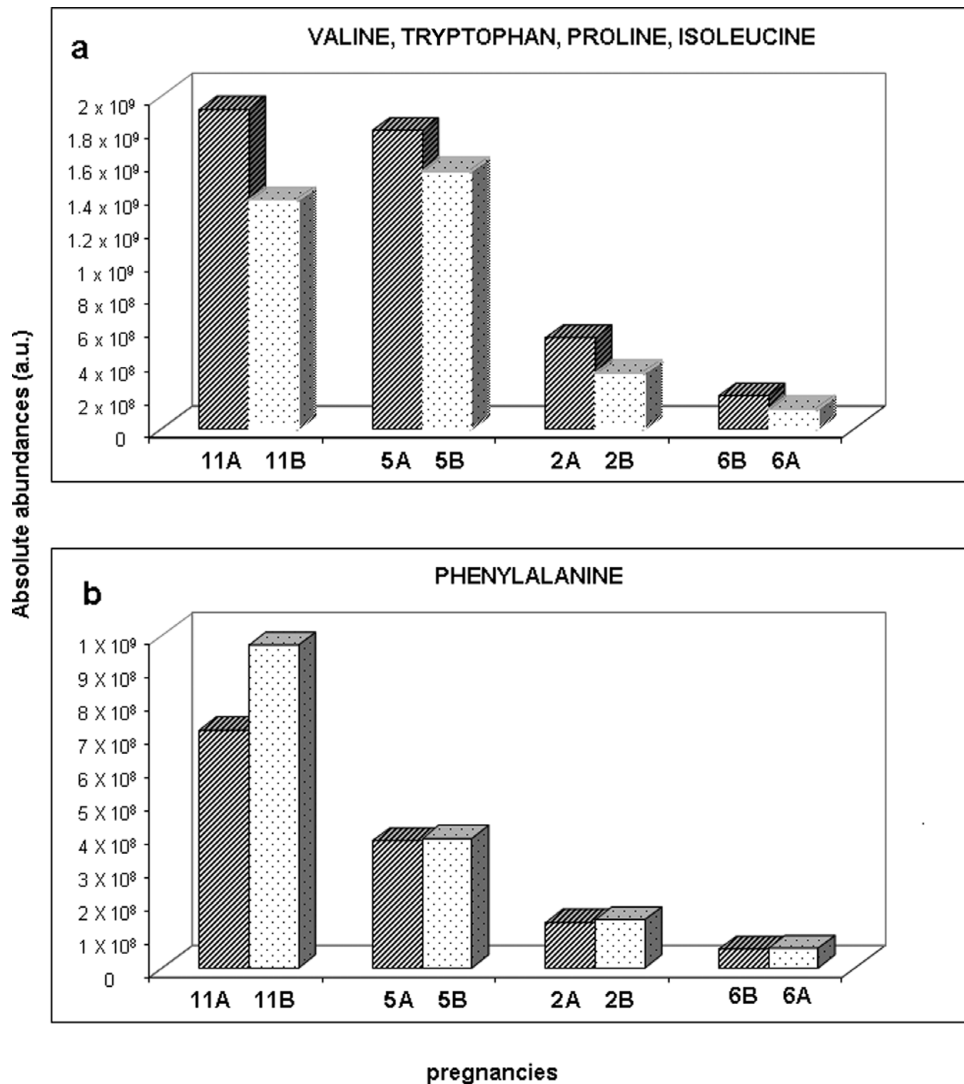
Although recent twin studies have taken into account the effect of intrauterine environment on fetal development trajectory using regression analyses and a between-twin coefficient (Carlin et al., 2005), few studies have compared the long-term health outcomes within twin pairs, and it is

still not clear whether the heavier twin in the pair has better health outcomes than the lower one (Phillips et al., 2001). In the literature, there are conflicting data concerning the relationship between IUGR, postnatal growth, and long-term outcomes (Bleker et al., 1995; De Matteo et al., 2008; McMillen & Robinson, 2005; Ross et al., 2005; Westwood et al., 2001).

Intima-media thickness is currently considered to be one of the earliest morphological markers of plaque formation and atherosclerosis in IUGR fetuses and neonates (Cosmi et al., 2009; Järvisalo et al., 2001; Koklu et al., 2007; Litwin & Niemirska, 2008; McGill et al., 2000; Skilton, 2008; Zanardo et al., 2011, 2013).

We have previously described the ultrasound-based measurement of abdominal aIMT in utero in IUGR single fetuses characterized by an EFW below the 10th percentile and $PI > 2 SD$, presenting persisting aortic wall thickening and microalbuminuria at the 18-month follow-up. Moreover, a microscopic observation of abdominal aortic walls of stillbirth IUGR fetuses confirmed that there is intima thickening (aIT) and the presence of inflammatory elements, such as macrophages and activated endothelial cells, even in amniotic fluid, indicating that intrauterine growth restriction is a potential marker of atherosclerosis development and may be associated with an increased risk of adult onset diseases (Cosmi et al., 2009; Lo Vasco et al., 2011, 2012; Zanardo et al., 2011).

Although the effect of IUGR on vascular function, assessed using indicators of endothelial damage such as the aIT, has previously been studied in dichorionic and

**FIGURE 2**

A comparison of the sera levels of amino acids valine, tryptophan, proline, isoleucine (a), and phenylalanine (b) between four sIUGR neonates with abnormal Doppler (Group 1, dotted bars) and four AGA controls (Group 3, striped bars). Each subject was considered as an independent statistical unit. As indicated in the text, a statistical test was performed (Student's *t* test for paired data), but due to the low sample size and the large dispersion of data it did not reach a statistically significant difference.

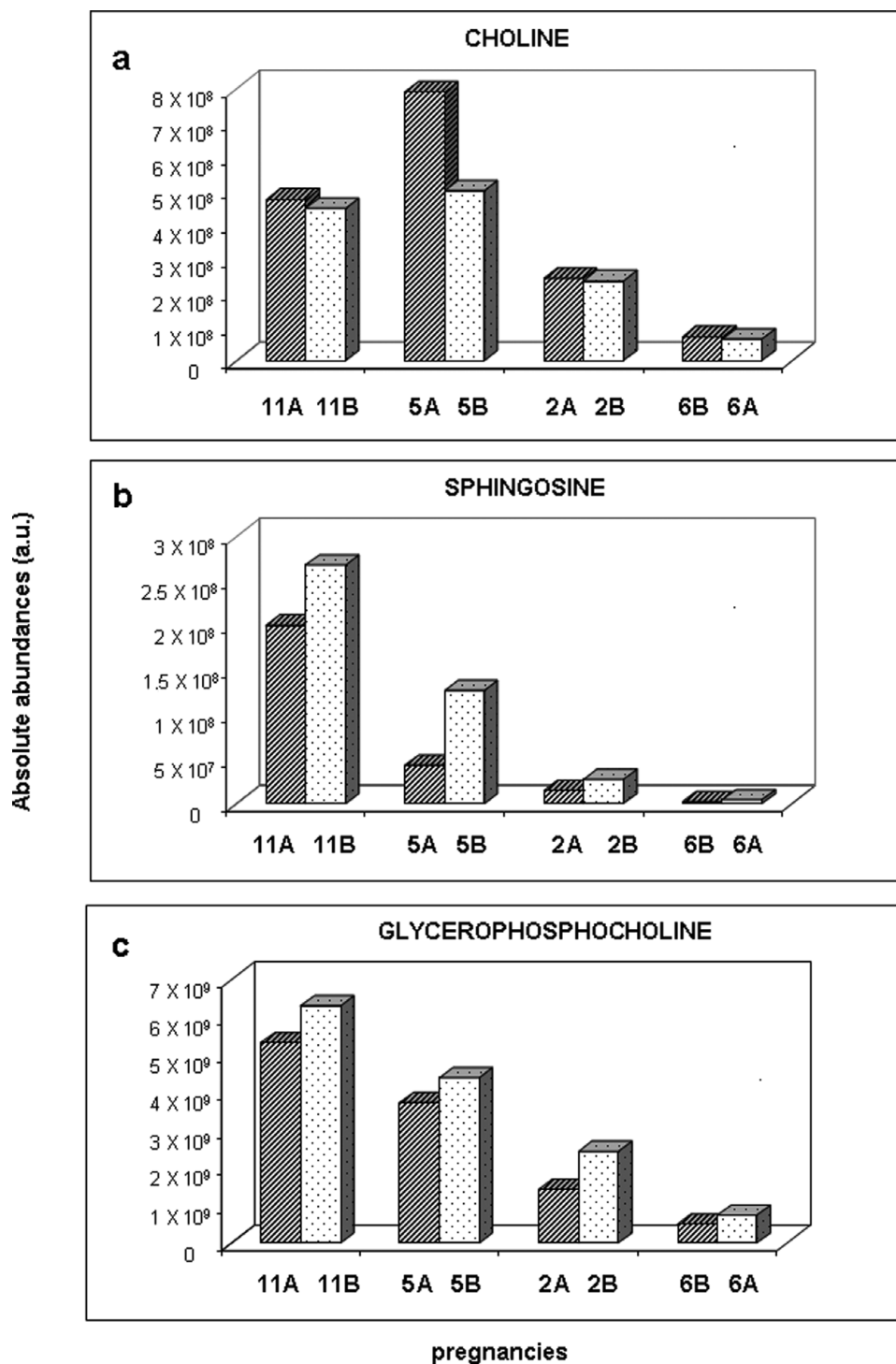
monochorionic twin pregnancies (Dwyer et al., 1999; Poulter et al., 1999), this is the first study conducted in a population of sIUGR monochorionic twins with and without Doppler alterations (Visentin et al., 2013). Our data show that low birth weight and Doppler umbilical artery vasculopathy in MDCA twins are accompanied by a higher aIT, compared with AGA co-twins and sIUGR neonates without Doppler alterations. This evidence suggests that a grading of endothelial alterations might exist, and that low birth weight and UA Doppler modifications could be used as predictors of the severity of the vasculopathy.

In light of the above, we have analyzed cord blood metabolomic profiles in sIUGR twins with and without UA Doppler abnormalities in order to deepen the inves-

tigation of the molecular mechanisms behind endothelial alterations and IUGR pathogenesis.

In previous studies conducted on singleton pregnancies, an upregulation of the amino acids phenylalanine, tryptophan, and glutamate was found in IUGR samples compared with AGA, suggesting a role of placental transport and metabolism in IUGR pathogenesis (Favretto et al., 2012; Matthews et al., 1998; Morris et al., 1995; Paolini et al., 2001).

In this investigation, a trend for increase of phenylalanine and a trend for downregulation of valine, isoleucine, tryptophan, and proline were found in sIUGR twin fetuses with UA Doppler abnormalities compared with control co-twins (no statistical significance could be reached due to the


FIGURE 3

A comparison of the sera levels of choline (a), sphingosine (b), and glycerophosphocholine (c) between four sIUGR neonates with abnormal Doppler (Group 1, dotted bars) and four controls (Group 3, striped bars). Each subject was considered as an independent statistical unit. As indicated in the text, a statistical test was performed (Student's *t* test for paired data), but due to the low sample size and the large dispersion of data it did not reach a statistically significant difference.

small number of subjects). Bajoria et al. (2001) described the selective reduction of phenylalanine in cord blood of MC twin pregnancies such as of other essential (valine, isoleucine, leucine, and arginine) and non-essential (tau-

rine, serine, glycine, tyrosine, and aspartic acid) amino acids by HPLC. The data of Bajoria and those of our investigation support the hypothesis that IUGR affecting one of the MC twins could be caused by an impaired placental

transport of amino acids rather than by an intertwin trans-fusion (Bajoria et al., 2001).

Additionally, a trend for increase of sphingosine was found in sIUGR twin neonates with UA Doppler alterations compared with AGA co-twins. Sphingosine is an aliphatic amino alcohol component of all sphingoglycolipids, most of which are localized on the cell surface and are involved as second messengers in a variety of cellular signaling pathways such as for cellular growth, differentiation, and migration. Sphingosine 1-phosphate, generated from sphingomyelin, is also expressed in the cardiovascular system and may be involved in the pathophysiology of diseases associated with endothelial dysfunction (Alewijjnse & Peters, 2008; Mulders et al., 2009; Spijkers et al., 2010). Because of their vasoactive properties, sphingolipids could play a pivotal role in hypertension, exemplified by the fact that the pharmacological modulation of vascular sphingoglycolipid composition in animal models induces pronounced transient contractile responses in several vessels; in particular, an elevation of ceramide, precursor of sphingosine, might induce a marked endothelium-dependent release of thromboxane A₂, involved in hypertension, and exocytosis of coagulation, pro-inflammatory and vasoactive substances (Chatterjee, 1998; Daum et al., 2009; Fenger et al., 2011; Spijkers et al., 2011).

In this study, increased glycerophosphocholine (GPC) sera concentrations were found in sIUGR Group 1 twins, compared with AGA co-twins. This finding could be explained by the need for the sIUGR fetus to use energy reserves due an insufficient supply. It is well known, indeed, that when energy requirements exceed energy availability, body fat is mobilized as a physiological mammalian process. Excessive mobilization leads to metabolic stress, which is thought to play a major role in the increased occurrence of food intake (Easter et al., 1971).

Although our study is limited by the very small sample size (partially explained by the rarity of the targeted condition in MDCA twins), and by the lack of cross-sectional observations that did not permit a longitudinal monitoring of the affected neonates, we believe that both echocardiography and metabolomic data could provide a good starting point for future more in-depth investigations of sIUGR in MDCA fetuses.

Conclusions

This study showed that aortic wall thickening is higher in sIUGR twins with UA Doppler velocimetry alterations than in sIUGR twins without UA Doppler anomalies or AGA co-twins. Moreover, an upregulation of serum phenylalanine, sphingosine, and GPC levels in sIUGR with UA Doppler waveforms was observed compared with AGA co-twins. Although no statistical significance was reached for metabolomic data due to the small number of investigated cases, we believe that our pilot study represents a valid start-

ing point for future in-depth metabolomic and proteomic investigations of sIUGR in MDCA fetuses.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/thg.2013.33>.

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