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Metabolomic patterns in second-trimester amniotic fluid and maternal serum associated with fetal trisomy 21

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Abstract

Metabolomic patterns in second-trimester amniotic fluid and maternal serum associated with fetal trisomy 21

By Susan Taylor Fischer

Introduction. Trisomy 21, otherwise known as Down syndrome, is a genetic disorder characterized by the presence of three copies of chromosome 21 and is one of the most prevalent chromosomal disorders in the United States. Trisomy 21 is associated with several medical conditions and birth defects including cognitive impairment and congenital heart defects, yet the biological mechanisms driving the presentation of associated phenotypes remain largely unknown. Metabolomic analysis of a large set of paired second-trimester maternal serum and amniotic fluid samples was performed with the objective of (i) further elucidating the fetal metabolic fingerprint associated with trisomy 21 at mid-pregnancy and (ii) investigating whether metabolic pathways dysregulated in trisomy 21 fetuses offer potential mechanisms of disorder-associated phenotypes.

Methods. Untargeted, high-resolution metabolomic analysis using a dual liquid chromatography setup was performed on 39 pairs of maternal serum and amniotic fluid samples from trisomy 21 pregnancies and 81 control sample pairs. Discriminatory features were identified in both biofluids using partial least squares discriminant analysis and variable importance in projection scores after adjusting for covariates and used as input for metabolic pathway enrichment analysis using the program Mummichog.

Results. Variable selection and subsequent pathway analysis of the amniotic fluid features detected in this study produced a complex and extensive set of perturbations associated with trisomy 21. While results indicated dysregulation of multiple pathways related to lipid metabolism, nucleotide metabolism, and amino acid metabolism, vitamin B3 metabolism ($p = 0.001$) was shown to be the most significantly affected pathway in amniotic fluid. Glycine, serine, alanine, and threonine metabolism was significantly perturbed in both biological matrices of trisomy samples.

Conclusions. Results revealed a broad array of metabolic perturbations in second-trimester trisomy 21 amniotic fluid and offered novel insight into possible fetal origins of the cognitive impairment and age-related neurodegeneration frequently observed with the disorder. The untargeted analytical platform has laid a foundation for follow-up targeted studies to confirm metabolic associations of interest and their role in phenotypic outcome pathogenesis.

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[1]Portions of the methods and results presented here were drawn from a manuscript in preparation for submission tentatively titled "Metabolic perturbations in second-trimester amniotic fluid associated with maternal exposure to secondhand smoke" by Fischer et al.

Contents

List of Abbreviations

AD	Alzheimer's disease
BCR	Balanced classification rate
BD	Becton-Dickinson
C_{18}	Octadecyl carbon chain
CBL	Clinical Biomarkers Laboratory, Emory University, Atlanta, GA, USA
CHD	Congenital heart defects
CV	Cross-validation
DS	Down syndrome
FC	Fold change
GGC	Greenwood Genetic Center, Greenwood, SC, USA
HCA	Hierarchical cluster analysis
HILIC	Hydrophilic interaction liquid chromatography
HMDB	Human Metabolome Database
IQR	Interquartile range
KEGG	Kyoto Encyclopedia for Genes and Genomes
LC-MS	Liquid chromatography-mass spectrometry
m/z	Mass-to-charge ratio
PCA	Principal component analysis
PLSDA	Partial least squares discriminant analysis
ppm	Parts per million
RT	Retention time
rpm	Revolutions per minute
SD	Standard deviation
SST	Serum separator tubes

SVM	Support vector machine
T21	Trisomy 21
US	United States
VIP	Variable importance in projection

1 Introduction

1.1 Problem Statement

Down syndrome (DS) is a genetic disorder characterized by the presence of a third chromosome 21, or trisomy 21. Technological advances in genomic analysis have opened the door for detailed examination of perturbed gene expression patterns associated with disease conditions of interest, including those associated with chromosomal abnormalities like trisomy 21 (Antonarakis, 2017). There is a growing literature on dysregulated biological pathways associated with an extra chromosome 21, but much remains to be learned with respect to the altered trisomy 21 fetal metabolism and its effects on known clinical conditions common to individuals with DS. Recent studies of amniotic fluid samples have conducted targeted analyses of metabolites with differential abundance among pregnancies with DS (Amorini et al., 2012; Charkiewicz, Blachnio-Zabielska, Zbucka-Kretowska, Wolczynski, & Laudanski, 2015, for example). However, there remains a need for studies aimed at defining the metabolome-wide alterations in a developing fetus associated with trisomy 21 and DS-related outcomes. Much of the current literature is limited by targeted quantification of select compounds, low sample size, or the specific goal of establishing reliable prenatal diagnostic biomarkers. Further investigation into the global metabolic consequences, both in the fetal and maternal compartments, of trisomy 21 during the earlier stages of pregnancy is needed.

1.2 Purpose Statement

The purpose of this study is to investigate the extent of differential biochemical composition between second-trimester amniotic fluid of fetuses with confirmed trisomy 21 compared to selected euploid controls through untargeted, high-resolution metabolomic analysis. Discovery of metabolite distributions unique to the trisomy 21 samples will then allow for mapping of discriminatory features to known metabolic pathways. The paired maternal serum sam-

ples allow investigation of the pronounced changes in the second-trimester fetal metabolomic profile related to trisomy 21 status as possible results of maternal environmental exposures or genetic variation. Also, any significantly enriched metabolic pathways in the amniotic fluid of trisomy 21 fetuses relative to controls can be further examined within the context of known DS-associated medical conditions, such as congenital heart defects (Freeman et al., 2008).

1.3 Significance Statement

Considering DS is one of the most prevalent chromosomal disorders in the United States (Parker et al., 2010), the results of this study will have important clinical implications. The unique bank of paired maternal serum and amniotic fluid samples available in this study, in combination with the untargeted analytic workflow employed, has potential to shed light on previously undetected metabolic perturbations associated with trisomy 21 during a period of particular developmental importance to a growing fetus. Furthermore, the outcomes observed here will help shape future studies of the prenatal exposome exploring the relationship between fetal and maternal environmental exposures. The next step in understanding how environmental exposures explain the wide variation in trisomy 21-associated clinical consequences will be informed by this study. Finally, the understanding of perturbed metabolic pathways in trisomy 21 fetuses will help identify potential modes of intervention for DS-associated conditions, including prevention of specific environmental exposures and possible prenatal therapeutic treatments.

1.4 Definition of Terms

- The term "feature" refers to a unique ion resolved by liquid chromatography-mass spectrometry, identified by mass-to-charge ratio and retention time. Feature intensity is a measure of ion abundance in a given biological sample and is determined by chromatographic peak integration (Go, Walker, Soltow, et al., 2015).

2 Review of Literature

Trisomy 21 is a genetic disorder characterized by the presence of three copies of chromosome 21 instead of the standard homologous pair of two chromosomes. The terms trisomy 21 and Down syndrome (DS) are often used interchangeably, while some prefer to distinguish DS as the collective phenotypic consequence of complete or incomplete trisomy of chromosome 21 (Borelli et al., 2015). This phenotypic consequence encompasses a variety of physical traits, medical conditions, and birth defects associated with DS, the most prominent being cognitive impairment, hypotonia, and congenital heart defects (CHD) (Karmiloff-Smith et al., 2016). Estimates from 2004-2006 national surveillance data across the United States (US) estimated a DS prevalence of 1 in 691 live births, corresponding to an estimated annual incidence count of approximately 6000 infants. Trisomy 21 is considerably more common than other chromosomal anomalies, including trisomy of chromosomes 13 or 18 (Parker et al., 2010). Worldwide live birth incidence estimates are lower than those found in the US, fluctuating around 1 in every 1000 live births ("Genes and chromosomal diseases", 2017).

As previously mentioned, DS is associated with a wide array of phenotypic features. To identify genes associated with these features, investigators have examined the effect of the full trisomy 21, while others have mapped features to specific chromosome regions among more rare individuals who have only partial duplication of chromosome 21 (Korenberg et al., 1994). While certain facial attributes, intellectual disability, and hypotonia are generally characteristic of DS, there is marked heterogeneity in the presentation of many associated characteristics. For example, a study from the National Down Syndrome Project reported significant variability in atrioventricular septal defect incidence among different ethnic and sex DS subpopulations (Freeman et al., 2008). Such variation in incidence and intensity of DS-linked traits, along with phenotype-specific mapping to select subregions of chromosome 21, illustrate the challenge faced by researchers looking to elucidate the biological mechanisms

of DS-associated phenotypes.

Gene expression studies and a limited but growing number of recent metabolomic analyses of different biofluids from DS individuals have begun to address such questions. Gene expression studies comparing second-trimester amniotic fluid cell-free fetal mRNA from individuals with DS to reference samples from seven chromosomally-normal (euploid) controls pointed to perturbations in several pathways (Hui et al., 2012; Slonim et al., 2009). In one study with analysis showing particular emphasis on nervous system development, investigators found many differentially expressed genes (Hui et al., 2012). For example, SOX11 is a transcription factor that plays a critical role in the regulation of sensory neuron axonal growth and was significantly down-regulated in fetuses with trisomy 21 compared to controls. In an earlier study of the same samples, investigators identified pathways related to oxidative stress that were perturbed in DS fetuses (Slonim et al., 2009). This conclusion is in agreement with another study of lipid peroxidation markers in 10 DS and 79 control amniotic fluid samples (Perrone et al., 2007). A review of evidence for altered protein expression in the fetal brain of individuals with DS further noted elevated activity of the superoxide dismutase gene, a gene mapped to chromosome 21 (Engidawork & Lubec, 2001). Despite the implication of oxidative stress in DS pathology, further research explicating the molecular mechanisms behind such pathway alterations and severe DS outcomes is needed.

Consistent with the findings above, a metabolomic investigation of maternal blood samples from mothers with DS-positive fetuses taken during the first trimester identified several potential DS biomarkers related to oxidative stress (Bahado-Singh et al., 2013). Analysis of maternal biofluids is important for improving trisomy 21 screening protocols in the first and second trimesters, which currently include measurement of select serum markers, such as human chorionic gonadotropin and alpha-fetoprotein, coupled with ultrasound (Nicolaides, 2011). A targeted study of sphingolipids in both maternal plasma and amniotic fluid from 10 DS pregnancies indicated differential levels of certain ceramides in DS-positive samples

(Charkiewicz et al., 2015). A metabolomic study of maternal urine collected during the second trimester further revealed unique DS metabolic profiles related to energy, nucleotide, and amino acid metabolism (Diaz et al., 2013). Thus, established metabolite biomarkers from targeted and untargeted metabolomic studies of amniotic fluid and other biofluids have provided a strong case for the potential value in additional high-resolution analyses of a larger number of paired maternal serum and amniotic fluid samples from pregnancies with DS. Importantly, amniotic fluid is a biological matrix that has been shown to strongly correlate with fetal anomalies and malformations (Amorini et al., 2012).

The growing popularity of metabolomic approaches across many academic disciplines can be attributed to multiple factors, including advances in liquid chromatography-mass spectrometry (LC-MS) technology that have allowed for improved mass resolution and lowered costs of analysis (Jones, 2016) and a desire for discovery of reliable disease biomarkers across a variety of biofluid sample types. Untargeted metabolomics allows for the detection of large-scale metabolic signatures associated with particular phenotypes. Use of these data as input to pathway enrichment analysis programs such as Mummichog (Li et al., 2013) allows for simultaneous feature annotation and pathway mapping by use of advanced computational algorithms.

While advances in instrumentation have enabled high-quality quantification of metabolites, there does not appear to be a common statistical approach deemed universally optimal for untargeted metabolomics data. Even within the context of simple binary disease outcomes like trisomy 21, the high dimensionality of the resulting data often requires application of complex analytical techniques (Madsen, Lundstedt, & Trygg, 2010). As such, a variety of univariate and multivariate statistical techniques have been used with the aim of identifying metabolite biomarkers capable of reliably discriminating subjects with DS from their non-DS counterparts. Here, the term univariate describes methods that analyze each feature independently, whereby the technique is repeated for each feature, assumes independence

between intensities of detected features, and requires correction for multiple testing. Multivariate methods are performed on the collective set of all detected features, or those remaining following preprocessing. In contrast to exploratory unsupervised methods, supervised multivariate methods incorporate knowledge of sample class or phenotypic identity to find significantly associated features (Alonso, Marsal, & Julia, 2015).

Both linear regression and logistic regression are often employed in the context of binary sample phenotypes, as these models can easily allow adjustment of data for potential confounders. Such univariate regression approaches do not, however, account for the possible correlations among intensities of detected features (Tzoulaki, Ebbels, Valdes, Elliott, & Ioannidis, 2014). Feature selection by means of partial least squares discriminant analysis (PLSDA), a supervised, multivariate statistical technique that seeks to maximize the covariance between class membership (e.g., DS case-control status) and the intensity profiles of the samples (Wold, Sjostrom, & Eriksson, 2001), is among the most frequently used methods in metabolomics for classification. Yet, its inability to adjust for any additional covariates is a key limitation of the approach. It is for this reason that a combination of univariate and multivariate statistical methods are often recommended in the metabolomics feature selection process. In one proposed approach, adjusted feature intensities are first obtained as residuals from linear regression models of relevant covariates and then used as input for multivariate modeling (Grapov, 2013).

3 Methodology

3.1 Study Samples

Paired amniotic fluid and maternal serum samples ($n = 120$) were obtained from the Greenwood Genetic Center (GGC, Greenwood, SC, USA). Samples were collected from mothers in the southeastern US who underwent prenatal screening during mid-pregnancy. The amniotic fluid samples were collected during amniocentesis from fetuses with a confirmed trisomy 21 (T21) karyotype (47,XY,+21 or 47,XX,+21; $n = 39$) and fetuses with a confirmed normal karyotype (46,XY or 46,XX; $n = 81$), with the latter set serving as control samples. Samples were previously stored as GGC clinical lab discards.

Serum and amniotic fluid samples were collected on-site at the facility of the mother's referring physician or laboratory and transported at ambient temperature to the prenatal testing laboratory at GGC either same day or overnight. Appropriate blood collection vials were used for maternal serum collection, including Becton-Dickinson (BD) red top vacutainer tubes, BD vacutainer red/black top serum separator tubes (SST), and BD vacutainer gold top SST. Standard BD plastic syringes were used for amniotic fluid sample collection, and samples were transported in clear or amber polystyrene tubes to the GGC laboratory. Once received, samples were inspected for correct identification, sample type, and sample condition; each received a unique sample identification number in compliance with accession protocols.

Maternal serum samples were received in the prenatal screening laboratory at GGC as either isolated serum samples, removed from the red blood cell clot by centrifugation, or as clotted whole blood samples. Whole blood samples were centrifuged at 2200 revolutions per minute (rpm) for 10 minutes and the serum transferred to separate polypropylene vials for storage. Serum samples were stored at 2-8°C for up to 48 hours before diagnostic testing and then

at -20°C (± 10°C) for long-term storage. Amniotic fluid samples received in the cytogenetic laboratory were centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and frozen at -20°C (± 10°C) for additional clinical testing and long-term storage.

Upon selection for this study, the maternal serum and amniotic fluid samples were removed from long-term storage, thawed, and transferred to new vials. Aliquots were refrozen and shipped on dry ice to Emory University by overnight courier. Upon receipt, aliquots were thawed and prepared for metabolomic analysis. No personally identifiable information was provided for the samples. However, information on maternal age, gestational age at both serum and amniotic fluid collection, sample collection year, maternal race/ethnicity, and fetal gender was available.

3.2 High-resolution LC-MS

Sample preparation and treatment prior to LC-MS followed previously documented protocols (Soltow et al., 2013). Analysis was performed by research specialists in the Clinical Biomarkers Laboratory (CBL, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University, Atlanta, GA, USA). Serum samples were run as triplicate aliquots on a Thermo Scientific LTQ Velos Orbitrap mass spectrometer. Dual liquid chromatography allowed for data collection from HILIC and C_{18} columns. Analyses were performed with positive electrospray ionization mode, an injection volume of 10 μL, mass-to-charge ratio (m/z) scan range of 85 to 2000, and resolution of 60000 (full width at half maximum).

Serum samples were randomly assigned to and run in batches of 20. Pooled reference plasma samples were analyzed prior to and following each batch, enabling subsequent quality control and metabolite quantification as described in a previous study (Go, Walker, Liang, et al., 2015). Data extraction was performed with apLCMS (Yu, Park, Johnson, & Jones, 2009) and xMSanalyzer (Uppal et al., 2013). Analysis of amniotic fluid samples was performed

separate from analysis of serum samples but under identical protocols. Principal component analysis (PCA) was used for batch effect evaluation (Yang et al., 2008), and batch correction was performed where necessary using ComBat (Johnson, Li, & Rabinovic, 2007) and xMSanalyzer.

3.2.1 Feature Annotation

Resulting xMSanalyzer output was received from CBL, including data matrices of detected ion features, defined by accurate mass m/z and retention time (RT, seconds), and corresponding quantified ion intensities across samples. Identities of a subset of metabolites were confirmed by comparison of m/z and RT to previously established CBL standards (Go, Liang, et al., 2015; Go, Walker, Liang, et al., 2015; Jones et al., 2016).

As an initial investigation of the differences in the baseline metabolic makeup of the amniotic fluid samples and that of the maternal serum samples, additional tentative matches for all features to known metabolites in the Kyoto Encyclopedia for Genes and Genomes (KEGG) database (Kanehisa & Goto, 2000) were generated using xMSannotator (Uppal, Walker, & Jones, 2016). Search criteria restricted matches to two commonly observed adduct forms under positive ionization mode ($[M+H]^{1+}$, $[M+Na]^{1+}$) at ± 10 ppm mass tolerance. The unique KEGG compound identifiers of metabolite matches were mapped using the online tool KEGG Mapper to human reference pathways. These annotations were used strictly as a preliminary overview of the global biochemical composition of each sample type (serum, amniotic fluid). Interpretation is limited by exclusion of features not matched to any KEGG compounds, exclusion of features not mapped to a human reference pathway, overrepresentation of certain features matched to more than one KEGG compound, and classification of certain compounds under several pathways.

3.3 Statistical Analysis

Samples were assigned new identification numbers prior to statistical analysis. As no information on the presence or absence of DS-associated traits was available for subjects, all results and conclusions here are presented within the context of fetal T21 as opposed to DS. Additionally, all results and corresponding figures were generated in R (R Core Team, 2015) unless otherwise indicated.

For each detected feature, the non-zero intensities of technical replicates were averaged and then $\log_2$-transformed. Feature filtering was performed to retain only those with at least 50% non-missing values across all samples and at least 80% non-missing values within samples of either group (control, T21). PCA, an unsupervised learning method, was first performed on the preprocessed feature intensity matrix. The top three components were used to visualize the variation in feature intensity profiles across the study samples in a score plot (Jolliffe, 2002). Samples that showed extreme separation from other data points in the three-dimensional space were considered outliers and excluded from further analysis.

Clinical and demographic variables were summarized with descriptive statistics and compared between control and T21 subjects using the two-sided Pearson's Chi-squared test for categorical variables (maternal race/ethnicity, fetal gender) and the two-sided t-test for numerical variables (maternal age, gestational age at sample collection, collection year). For covariates that differed significantly between groups ($p < 0.05$), their global effect on metabolic profiles was investigated using the PCA model described above; score plots were again used to visually assess whether the spatial clustering or separation of data points was associated with the covariate(s) of interest.

Feature intensities in each of the four datasets (maternal serum [HILIC column, C_{18} column], amniotic fluid [HILIC column, C_{18} column]) were then adjusted for potential confounders

using a univariate regression approach. For each detected feature, a linear regression model was constructed with $\log_2$-transformed intensity as the response variable and the relevant covariate(s) that differed significantly between groups as predictors. The residuals were then used to construct intensity matrices linearly corrected for confounder effects prior to feature selection.

3.3.1 Feature Selection

Feature selection was conducted using partial least squares discriminant analysis (PLSDA), a supervised, multivariate statistical technique that seeks to maximize the covariance between group membership and intensity profiles of the samples (Wold et al., 2001). Results were visualized using two-dimensional score plots. The variable importance in projection (VIP) scores were used to identify the set of m/z features within each dataset with greatest contribution to the discriminatory model (Palermo, Piraino, & Zucht, 2009). These features were selected on the basis of VIP ≥ 2 (Bogdanov et al., 2008). The prediction performance of the PLSDA-selected features in accurately distinguishing the two groups was evaluated by 10-fold cross-validation (CV) and support vector machines (SVM); here, the discriminatory features from the PLSDA model with VIP ≥ 2 were used to build the binary SVM classifier prior to CV. The reduced intensity profiles corresponding to only the discriminatory features selected by PLSDA were used as input for two-way, unsupervised hierarchical cluster analysis. To determine the extent of overlap in PLSDA-selected features between datasets, m/z were compared using xMSanalyzer (Uppal et al., 2013) and a maximum difference threshold of ± 10 ppm for matches.

3.4 Pathway Enrichment Analysis

Pathway enrichment analysis and simultaneous annotation of discriminatory features was performed using the Python program Mummichog (Li et al., 2013). For each sample type (maternal serum, amniotic fluid), all m/z from HILIC and C_{18} analyses that met

feature filtering thresholds were combined to form a larger, more comprehensive background metabolome; this further established an enriched set of discriminatory features (VIP ≥ 2) for both serum and amniotic fluid. This approach can provide improved confidence in pathway selection when discriminatory features in the C_{18} and HILIC datasets are mapped to the same metabolic pathway by increasing pathway overlap size. Mummichog was run using human reference pathways from MetaFishNet as the network model (Li et al., 2010). Pathways with $p < 0.05$ were considered significantly enriched in association with fetal T21 status.

Mummichog output was further augmented by annotation of discriminatory features using xMSannotator (Uppal et al., 2016), first obtaining putative matches to known metabolites in the Human Metabolome Database (HMDB) (Wishart et al., 2007) on the basis of accurate mass m/z with a mass error threshold of ± 10 ppm. When searching HMDB, multiple adducts were considered: $[M+Na]^{1+}$, $[M+H]^{1+}$, $[M+H\text{-}H_2O]^{1+}$, $[M+ACN+H]^{1+}$, $[M+2Na\text{-}H]^{1+}$, $[2M+H]^{1+}$, $[M+2H]^{2+}$, $[2M+Na]^{1+}$, $[M+NH_4]^{1+}$, $[2M+ACN+H]^{1+}$, $[M+H\text{-}2H_2O]^{1+}$, $[M+ACN+Na]^{1+}$. Using xMSannotator's multilevel scoring algorithm to assign confidence levels to all annotations, levels 2 (medium confidence) and 3 (high confidence) were accepted. These annotations from xMSannotator, along with the confirmed metabolite matches identified in 3.2.1, were used to supplement Mummichog annotation of discriminatory features.

4 Results

4.1 Global Metabolic Profiles in Serum and Amniotic Fluid

High-resolution LC-MS detected 7807 features (C_{18} chromatography) and 12884 features (HILIC chromatography) in the maternal serum samples and 3828 and 8541 features, respectively, in the amniotic fluid samples. Feature matching using m/z, with a mass error threshold of ± 10 ppm, provided a rough idea of metabolic coverage similarity across columns. The majority of C_{18}-detected m/z were also found by HILIC separation (63.2% of serum; 65.3% of amniotic fluid), while the majority of HILIC m/z were unique and not detected by C_{18} separation (61.7% of serum; 70.8% of amniotic fluid). Similar feature matching between HILIC maternal serum and HILIC amniotic fluid datasets (± 10 ppm mass error threshold, ± 30 second RT threshold) showed 3918 common to both matrices. Nearly half of all amniotic fluid features detected by HILIC chromatography (45.9%) overlapped those detected in serum, and a similar proportion was seen for C_{18}-detected features (44.8%).

As a preliminary exploration of the global metabolic differences between the second-trimester serum and amniotic fluid samples under study, all detected m/z from the HILIC column were searched against known compounds in the KEGG database and mapped to human reference pathways in the KEGG database. HILIC-detected features were used due to greater coverage of that column across both sample types, as described above. Tentative matches for 6366 unique KEGG compounds were found in serum. Among these compounds, 188 KEGG human pathways were represented; specifically, 634 compounds were mapped to human metabolic pathways. In the amniotic fluid, detected features were tentatively matched to 5988 unique KEGG compounds; this set mapped to 200 distinct human pathways, and 648 compounds were mapped to reference human metabolic pathways. Among metabolic pathways, results showed baseline representation of amino acid and carbohydrate metabolism matches in the amniotic fluid slightly exceeded that of maternal serum (Fig. 1). Serum matches indicated

greater global representation of lipid metabolism, although this difference was again minimal.

Of the features detected in amniotic fluid by C_{18} and HILIC analysis, 3105 (81.1%)and 6362 (74.5%) passed the filtering criteria, that is they were present in (i) at least half of all samples and (ii) $\geq 80\%$ of all control samples or all T21 samples. Similar filtering of maternal serum data retained 5861(75.1%) and 9376 (72.8%) C_{18} and HILIC features, respectively. Variation in global metabolic profiles across samples was assessed by PCA score plots. As shown in Fig. 2A and Fig. 2B, T21 amniotic fluid samples did not cluster separately from control samples, indicative of similarity in metabolic profiles at least on the global level. However, one sample (S246, control) showing noticeable separation from other amniotic fluid samples in both C_{18} and HILIC models was identified as an outlier and excluded from further analysis.

4.2 Study Population

Characteristics of the study population ($n = 119$) following outlier removal are summarized in Table 1. All maternal serum samples were collected during the second trimester (15.0-22.0 weeks gestation). The mean ($\pm$ SD) gestational age at amniocentesis was 20.5 $\pm$ 3.1 weeks, ranging between 15.7 and 31.7 weeks. As only four of 119 amniotic fluid samples (2/39 T21 samples; 2/80 control samples) qualified as early third-trimester, results are presented within the framework of second trimester collection. On average, maternal serum collection preceded amniocentesis by close to three weeks (mean 3.1 ± 2.7); however, for two control subjects, amniotic fluid was collected one week prior to maternal serum collection.

Maternal race/ethnicity, fetal gender, gestational age at serum collection, gestational age at amniotic fluid collection, and maternal age did not differ significantly between control and T21 subjects ($p > 0.05$, Table 1). However, the T21 samples, collected between 2000 and 2014, were significantly older than the control set, collected between 2004 and 2014 ($p = 0.01$). This was due to a more limited supply of T21-postive GGC clinical discard samples and a greater availability of more recent control discards. PCA score plots further

revealed a noticeable association of amniotic fluid metabolic profiles with storage time after stratifying by fetal T21 status (Fig. 3A and Fig. 3B). Feature intensities were therefore adjusted for sample collection year by taking the residuals from linear regression models of $\log_2$-transformed intensity against collection year.

4.3 Untargeted Metabolomic Analysis of Amniotic Fluid

4.3.1 Feature Selection

Two-dimensional PLSDA score plots of amniotic fluid samples are provided in Fig. 4A (HILIC) and Fig. 4B (C_{18}). Both models achieved adequate separation of control and T21 samples. A stringent cutoff of VIP ≥ 2 identified 125 (C_{18}) and 220 (HILIC) discriminatory features. Manhattan plots of all VIP scores (Fig. 5A and Fig. 5B) show that most of the discriminatory features fell in the region of low molecular mass (<500 m/z). Using a mass error threshold of $\pm$10 ppm for matching, 27 common features were found between these two sets of discriminatory features, representing 12.3% of the HILIC set and 21.6% C_{18} set (Fig. 6A).

The 10-fold CV analysis, performed outside of PLSDA using significant features to first construct a SVM, assessed accuracy using balanced classification rate (BCR), an average measure of classification accuracy in each group useful when group sample sizes are unbalanced. Both sets of PLSDA-selected features effectively distinguished the T21 and control samples (Table 2). When sample labels were permuted and CV was repeated, the probability of the SVM correctly classifying samples was comparable to pure chance (mean permuted BCR = 50%).

Hierarchical cluster analysis (HCA) provided an alternate approach to visualizing similarity in intensity profiles of significant features in amniotic fluid. As shown in Fig. 7, HCA of HILIC discriminatory features resulted in two primary clusters, one with 92% T21 member-

ship and the other with 94% control membership. Cluster membership was more heterogeneous in HCA of C_{18} discriminatory features (Fig. 8). Three primary clusters are evident here: one consisting exclusively of control samples, one with 80% T21 membership, and another containing a mix of T21 and control samples. When comparing chromatography columns, the heat maps suggest that the HILIC-selected features show a more distinct and consistent expression pattern in T21 amniotic fluid samples relative to controls than those selected from the C_{18} dataset.

4.3.2 Pathway Analysis

Input of all 345 discriminatory features across columns to Mummichog for pathway analysis yielded 19 enriched metabolic pathways in the amniotic fluid of T21 pregnancies ($p < 0.05$). Those with at least four overlap metabolites (14) are presented in Table 3. In this table, pathway size represents the total number of metabolites, regardless of VIP score, mapped to a given pathway, while overlap size represents the number of those metabolites that were found to be significant by PLSDA. Results showed dysregulation of multiple pathways related to lipid metabolism (linoleate metabolism, fatty acid metabolism), nucleotide metabolism (pyrimidine metabolism, purine metabolism), and amino acid metabolism in amniotic fluid of T21 fetuses. Examples of perturbed amino acid pathways included methionine and cysteine metabolism and glycine, serine, alanine, and threonine metabolism. The two pathways showing greatest dysregulation in association with T21 were vitamin B3 metabolism ($p = 0.001$) and butanoate metabolism ($p = 0.002$).

A subset of representative features selected by PLSDA as best discriminating amniotic fluid of T21 fetuses relative to controls (VIP ≥ 2) are provided in Table 4, where fold change (FC) indicates difference in mean $\log_2$ intensity between T21 cases and controls. Positive FC values indicate greater average abundance among T21 samples, and metabolite superscripts indicate annotation source (confirmed identity, Mummichog, xMSannotator). Features matched by

Mummichog to stearidonic acid were among the top 10 significant features by VIP score in both chromatography columns, and features matched to palmitaldehyde were among the top 20 of both columns.

4.4 Untargeted Metabolomic Analysis of Maternal Serum

4.4.1 Feature Selection

The analytic workflow outlined above was repeated for metabolomic data derived from maternal sera. The PLSDA models yielded 324 (C_{18}) and 502 (HILIC) features meeting the significance threshold (VIP ≥ 2), and testing for overlap between chromatography columns showed 44 common discriminatory features, representing 8.8% of the HILIC set and 13.6% C_{18} set (Fig. 6B). The PLSDA score plots are included in Fig. 4C and Fig. 4D. In contrast to the amniotic fluid models, both serum models failed to achieve complete separation of serum samples from control and T21 pregnancies. Collectively, the features with greatest contribution to the PLSDA models in maternal serum also showed poorer predictive ability under 10-fold CV analysis (Table 2) compared to those from amniotic fluid. These results indicate an overall greater discriminatory power of selected amniotic fluid features in discerning fetal T21 status than those identified in corresponding maternal serum.

HCA of $\log_2$ intensity profiles revealed variable expression patterns of selected serum features within T21 samples (Fig. 9 and Fig. 10). However, both heat maps illustrate a unique and homogenous expression pattern of PLSDA-selected features among a smaller subset of T21 maternal serum cases. It is particularly interesting that the majority of the T21 subjects (13 of 15) whose sera clustered separately in the HILIC HCA were the same as those that clustered separately from other T21 cases in the C_{18} HCA. This would suggest an underlying connection, possibly genotypic, phenotypic, or environmental in nature, among this subset of mothers with T21 pregnancies. Follow-up Mann-Whitney U tests showed that the 13 mothers making up this unique cluster were significantly younger (median age = 27.0 ± 12.0) than

the other mothers with T21 pregnancies (33.5 ± 12.8; $p = 0.04$) while their serum samples were significantly older (median collection year = 2002 ± 3.0) than the others (2007 ± 4.8; $p < 0.01$).

4.4.2 Pathway Analysis

Mummichog analysis of all 826 serum features with VIP ≥ 2 yielded 15 enriched metabolic pathways in the maternal serum of T21 cases ($p < 0.05$), all of which contained at least four overlap metabolites (Table 3). Results indicated particular dysregulation of many amino acid pathways, the most affected being glycine, serine, alanine, and threonine metabolism ($p = 0.002$). This pathway was also found to be perturbed in T21 amniotic fluid ($p = 0.016$), and Mummichog results for this pathway in maternal serum are provided in Table 5. The serum of mothers with T21 pregnancies, in particular, showed increased levels of confirmed serine and threonine, while decreased levels of confirmed creatine were observed among cases. The difference in abundance of these metabolites between groups was not large in magnitude, however, as indicated by FC. In contrast, Mummichog annotations showed more pronounced decreases in matches to choline, methionine, and glycolic acid. The discriminatory serum metabolites mapped to this pathway, also referred to as the overlap metabolites, were largely distinct from those seen in the amniotic fluid with the exception of serine, which instead showed slightly lower abundance in T21 amniotic fluid relative to controls (Table 4).

The significantly enriched pathways in serum also overlapping those perturbed in the amniotic fluid samples of T21 subjects included butanoate metabolism, aspartate and asparagine metabolism, and arginine and proline metabolism; maternal serum from T21 pregnancies showed increased levels of confirmed proline (VIP = 2.64, FC = 0.62 [C_{18}]). Among the dysregulated metabolic pathways unique to serum samples, T21 maternal serum had elevated levels of confirmed tyrosine (VIP = 2.65, FC = 0.86 [C_{18}]), lysine (VIP = 3.32, FC = 0.95 [HILIC]), and decreased levels of glycerophosphocholine (VIP 2.62, FC = -0.92 [HILIC]).

5 Discussion

The growth of metabolomics in recent years has generated tremendous momentum in the detection, identification, and quantification of endogenous compounds across a variety of biological matrices. Such research is helping to establish a library of putatively normal "reference" metabolic profiles to which samples with conditions of interest can then be compared. The discovery of unique metabolic profiles holds great clinical importance not only in its ability to improve diagnostic screening for complex diseases, but particularly in its potential to illuminate the pathogenesis of developmental anomalies or unique morphological characteristics, such as those with ubiquitous or even variable prevalence among people with DS.

The data presented here are the result of an untargeted, high-resolution metabolomic analysis of paired second-trimester maternal serum and amniotic fluid samples from fetuses with and without confirmed trisomy of chromosome 21. This study was conducted in an effort to further explore the metabolic fingerprint associated with T21 during the second trimester of pregnancy. The first and second trimesters of pregnancy are a period of particular vulnerability for a developing fetus due to continued organ development and an immature detoxification system. Metabolomic profiling of amniotic fluid at this period of gestation can reveal fetal exposure to environmental chemicals and xenobiotics transferred from the maternal system via the placenta (Barr, Bishop, & Needham, 2007) and also serves as a suitable barometer of fetal health and development status (Graca et al., 2009).

An initial comparison of features resolved by HILIC chromatography in amniotic fluid samples to those resolved in the corresponding maternal serum samples demonstrated considerable overlap between the two biofluids. This observation makes sense given the sizable contribution of maternal plasma solutes to amniotic fluid composition in the first half of preg-

nancy. Fetal skin keratinization starts approximately half way through the second trimester and concludes around 25 weeks, after which the greatest contributor to the amniotic fluid composition is fetal urine (Underwood, Gilbert, & Sherman, 2005). Thus metabolic profiles of the amniotic fluid samples here should indeed be a reflection of both maternally-derived compounds as well as endogenous metabolites of the fetal compartment. Pathway mapping indicated slightly greater background representation of metabolites related to amino acid metabolism relative to maternal serum. A possible explanation may be offered by the passive diffusion of amino acids from fetal circulation into amniotic fluid through the permeable skin barrier (Jauniaux, Gulbis, & Gerloo, 1999).

Feature selection and subsequent pathway enrichment analysis of the amniotic fluid metabolic profiles in this study produced a complex and extensive set of perturbations associating with T21 classification. The differential abundance of select confirmed metabolites, as well as those annotated by Mummichog and xMSannotator, showed consistency with much of the existing literature on metabolic biomarkers of T21. Amniotic fluid of T21 fetuses showed decreased C16-Cer, as seen in a previous study of ceramides in 10 DS amniotic fluid samples taken at 15-18 weeks gestation (Charkiewicz et al., 2015). Furthermore, significant enrichment of several amino acid pathways was observed. In accordance with the results of a targeted assay of amino acids in T21 second-trimester amniotic fluid (Amorini et al., 2012), the data similarly showed decreased levels of glutamate, valine, and ornithine among T21 cases; in contrast, taurine was found here to be elevated in association with T21. Certain metabolites previously observed to be lower in concentration in amniotic fluid in association with fetal malformations (e.g., central nervous system, cardiac, pulmonary), including glucose, alanine, threonine, tyrosine, methionine, and phenylalanine (Graca et al., 2010), were negatively associated with T21, although none met the VIP threshold for significance. Considered collectively, these associations support in part the previous suggestions of dysregulated gluconeogenesis activity and the role of related amino acids in impaired fetal kidney

and neuronal development (Amorini et al., 2012).

Vitamin B3 metabolism was shown to be the most affected pathway in T21 amniotic fluid, with increased abundance of matches to alpha-D-ribose 1-phosphate and 1-methylnicotinamide among cases. Possible implications include altered energy metabolism and/or oxidative phosphorylation in the T21 fetal compartment. Existing literature has begun to document preliminary investigations into the association of nicotinate and nicotinamide metabolism with neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's (Sauve, 2008). AD, in particular, is highly over-represented in the adult DS population relative to non-DS individuals and individuals with intellectual disabilities (Grieco, Pulsifer, Seligsohn, Skotko, & Schwartz, 2015). A study of brain tissue from transgenic mice demonstrating the severe neuropathology of AD showed increased levels of nicotinamide (Qin et al., 2006). When considered in conjunction with decreased levels of two confirmed phosphatidylcholines, a class of compounds showing therapeutic promise in mitigating the severity of cognitive dysfunction and combating the onset of AD among individuals with DS (Moon et al., 2010), results of this study may provide particular insight into the fetal origins of the cognitive impairment and age-related neurodegeneration frequently observed with DS.

5.1 Strengths and Limitations

Sample size represents a key strength of this analysis. A literature search of comparable studies showed this sample set to be the largest collection of T21 amniotic fluid samples ever used, to investigator knowledge, for metabolomic profiling, let alone analyzed in conjunction with paired maternal serum samples. Furthermore, while the synthesis of dual chromatography data adds complexity to analysis, the expanded coverage ability offers many advantages. The differential detection coverage of columns, preferential detection of certain classes of chemicals between chromatography columns, and reduced cost of analysis with a dual chromatography setup underscores the value of this approach in comprehensive

metabolic profiling of the fetal exposome (Soltow et al., 2013; Jones, 2016).

While the volume of available T21 samples and employment of advanced LC-MS procedures are notable strengths, this study also has important limitations. For example, serum and amniotic fluid samples were run separately during LC-MS analysis, and this prevented the calculation of metabolite ratios between paired maternal and fetal samples. Additional limitations included incomplete demographic, maternal exposure, and clinical profiles of subjects whose samples were analyzed. Such information might have revealed defining characteristics of the subset of T21 mothers whose serum profiles clustered separately from other T21 subjects in HCA; it is currently unknown whether this clustering is the result of a clinically important unmeasured covariate, temporal variations in serum profiles related to storage time, or inconsistent sample collection or storage protocols. With respect to the amniotic fluid analysis, an important addition to the study would have been the inclusion of birth outcome data, most demonstrably those related to known DS-associated birth defects. Lastly, pathway enrichment analysis relied on putative annotations of many discriminatory metabolites for which confirmed m/z and RT data were not available.

5.2 Future Directions

Pathway enrichment results from analysis of T21 amniotic fluid suggested dysregulation of steroid hormone biosynthesis and showed pronounced difference in abundance of steroid glucuronide matches between groups. Follow-up confirmation of these metabolite identities by tandem mass spectrometry would help illuminate the role of steroid hormones in T21 fetal development (Kaludjerovic & Ward, 2012), possibly within the context of endocrine abnormalities often observed in DS individuals (Campos & Casado, 2015). Results also indicated perturbation of pathways related to lipid metabolism. Features mapped to linoleate metabolism were increased in the amniotic fluid of T21 fetuses, while the FC of features mapped to fatty acid pathways showed inconsistent directionality between T21 groups. Fu-

ture targeted lipid profiling may elucidate the metabolic consequence of T21 in a developing fetus within these pathways, as well as confirm the associations observed with select acylcarnitines.

References

Alonso, A., Marsal, S., & Julia, A. (2015). Analytical methods in untargeted metabolomics: state of the art in 2015. *Front Bioeng Biotechnol*, *3*, 23.

Amorini, A. M., Giorlandino, C., Longo, S., D'Urso, S., Mesoraca, A., Santoro, M. L., ... Tavazzi, B. (2012). Metabolic profile of amniotic fluid as a biochemical tool to screen for inborn errors of metabolism and fetal anomalies. *Mol Cell Biochem*, *359*(1-2), 205-16.

Antonarakis, S. E. (2017). Down syndrome and the complexity of genome dosage imbalance. *Nat Rev Genet*, *18*(3), 147-163.

Bahado-Singh, R. O., Akolekar, R., Mandal, R., Dong, E., Xia, J., Kruger, M., ... Nicolaides, K. (2013). Metabolomic analysis for first-trimester Down syndrome prediction. *Am J Obstet Gynecol*, *208*(5), 371 e1-8.

Barr, D. B., Bishop, A., & Needham, L. L. (2007). Concentrations of xenobiotic chemicals in the maternal-fetal unit. *Reprod Toxicol*, *23*(3), 260-6.

Bogdanov, M., Matson, W. R., Wang, L., Matson, T., Saunders-Pullman, R., Bressman, S. S., & Flint Beal, M. (2008). Metabolomic profiling to develop blood biomarkers for Parkinson's disease. *Brain*, *131*(Pt 2), 389-96.

Borelli, V., Vanhooren, V., Lonardi, E., Reiding, K. R., Capri, M., Libert, C., ... Wuhrer, M. (2015). Plasma N-glycome signature of Down syndrome. *J Proteome Res*, *14*(10), 4232-45.

Campos, C., & Casado, A. (2015). Oxidative stress, thyroid dysfunction & Down syndrome. *Indian J Med Res*, *142*(2), 113-9.

Charkiewicz, K., Blachnio-Zabielska, A., Zbucka-Kretowska, M., Wolczynski, S., & Laudanski, P. (2015). Maternal plasma and amniotic fluid sphingolipids profiling in fetal Down syndrome. *PLoS One*, *10*(5), e0127732.

Diaz, S. O., Barros, A. S., Goodfellow, B. J., Duarte, I. F., Galhano, E., Pita, C., ... Gil, A. M. (2013). Second trimester maternal urine for the diagnosis of trisomy 21 and prediction of poor pregnancy outcomes. *J Proteome Res*, *12*(6), 2946-57.

Engidawork, E., & Lubec, G. (2001). Protein expression in Down syndrome brain. *Amino Acids*, *21*(4), 331-61.

Freeman, S. B., Bean, L. H., Allen, E. G., Tinker, S. W., Locke, A. E., Druschel, C., ... Sherman, S. L. (2008). Ethnicity, sex, and the incidence of congenital heart defects: a report from the National Down Syndrome Project. *Genet Med*, *10*(3), 173-80.

Genes and chromosomal diseases. (2017). *World Health Organization*. Retrieved Feb 6, 2017, from `http://who.int/genomics/public/geneticdiseases/en/index1.html`

Go, Y. M., Liang, Y., Uppal, K., Soltow, Q. A., Promislow, D. E., Wachtman, L. M., & Jones, D. P. (2015). Metabolic characterization of the common marmoset (Callithrix jacchus). *PLoS One*, *10*(11), e0142916.

Go, Y. M., Walker, D. I., Liang, Y., Uppal, K., Soltow, Q. A., Tran, V., ... Jones, D. P. (2015). Reference standardization for mass spectrometry and high-resolution metabolomics applications to exposome research. *Toxicol Sci*, *148*(2), 531-43.

Go, Y. M., Walker, D. I., Soltow, Q. A., Uppal, K., Wachtman, L. M., Strobel, F. H., ...

Jones, D. P. (2015). Metabolome-wide association study of phenylalanine in plasma of common marmosets. *Amino Acids*, *47*(3), 589-601.

Graca, G., Duarte, I. F., Barros, A. S., Goodfellow, B. J., Diaz, S., Carreira, I. M., ... Gil, A. M. (2009). (1)H NMR based metabonomics of human amniotic fluid for the metabolic characterization of fetus malformations. *J Proteome Res*, *8*(8), 4144-50.

Graca, G., Duarte, I. F., Barros, A. S., Goodfellow, B. J., Diaz, S. O., Pinto, J., ... Gil, A. M. (2010). Impact of prenatal disorders on the metabolic profile of second trimester amniotic fluid: a nuclear magnetic resonance metabonomic study. *J Proteome Res*, *9*(11), 6016-24.

Grapov, D. (2013). Covariate adjustement for PLS-DA models. *Creative Data Solutions*. Retrieved Jan 6, 2017, from `https://imdevsoftware.wordpress.com/2013/01/13/covariate-adjustement-to-simplify-and-improve-inference-in-multi-factorial-experimental-designs/`

Grieco, J., Pulsifer, M., Seligsohn, K., Skotko, B., & Schwartz, A. (2015). Down syndrome: cognitive and behavioral functioning across the lifespan. *Am J Med Genet C Semin Med Genet*, *169*(2), 135-49.

Hui, L., Slonim, D. K., Wick, H. C., Johnson, K. L., Koide, K., & Bianchi, D. W. (2012). Novel neurodevelopmental information revealed in amniotic fluid supernatant transcripts from fetuses with trisomies 18 and 21. *Hum Genet*, *131*(11), 1751-9.

Jauniaux, E., Gulbis, B., & Gerloo, E. (1999). Free amino acids in human fetal liver and fluids at 12-17 weeks of gestation. *Hum Reprod*, *14*(6), 1638-41.

Johnson, W. E., Li, C., & Rabinovic, A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics*, *8*(1), 118-27.

Jolliffe, I. (2002). *Principal component analysis* (2nd ed.). New York, NY: Springer New York.

Jones, D. P. (2016). Sequencing the exposome: A call to action. *Toxicol Rep*, *3*, 29-45.

Jones, D. P., Walker, D. I., Uppal, K., Rohrbeck, P., Mallon, C. T., & Go, Y. M. (2016). Metabolic pathways and networks associated with tobacco use in military personnel. *J Occup Environ Med*, *58*(8 Suppl 1), S111-6.

Kaludjerovic, J., & Ward, W. E. (2012). The interplay between estrogen and fetal adrenal cortex. *J Nutr Metab*, *2012*, 837901.

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*, *28*(1), 27-30.

Karmiloff-Smith, A., Al-Janabi, T., D'Souza, H., Groet, J., Massand, E., Mok, K., ... Strydom, A. (2016). The importance of understanding individual differences in Down syndrome. *F1000Res*, *5*.

Korenberg, J. R., Chen, X. N., Schipper, R., Sun, Z., Gonsky, R., Gerwehr, S., ... et al. (1994). Down syndrome phenotypes: the consequences of chromosomal imbalance. *Proc Natl Acad Sci U S A*, *91*(11), 4997-5001.

Li, S., Park, Y., Duraisingham, S., Strobel, F. H., Khan, N., Soltow, Q. A., ... Pulendran, B. (2013). Predicting network activity from high throughput metabolomics. *PLoS Comput Biol*, *9*(7), e1003123.

Li, S., Pozhitkov, A., Ryan, R. A., Manning, C. S., Brown-Peterson, N., & Brouwer, M. (2010). Constructing a fish metabolic network model. *Genome Biol*, *11*(11), R115.

Madsen, R., Lundstedt, T., & Trygg, J. (2010). Chemometrics in metabolomics – a review in human disease diagnosis. *Anal Chim Acta*, *659*(1-2), 23-33.

Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behav Neurosci*, *124*(3), 346-61.

Nicolaides, K. H. (2011). Screening for fetal aneuploidies at 11 to 13 weeks. *Prenat Diagn*, *31*(1), 7-15.

Palermo, G., Piraino, P., & Zucht, H. D. (2009). Performance of PLS regression coefficients in selecting variables for each response of a multivariate PLS for omics-type data. *Adv Appl Bioinform Chem*, *2*, 57-70.

Parker, S. E., Mai, C. T., Canfield, M. A., Rickard, R., Wang, Y., Meyer, R. E., ... National Birth Defects Prevention Network (2010). Updated national birth prevalence estimates for selected birth defects in the United States, 2004-2006. *Birth Defects Res A Clin Mol Teratol*, *88*(12), 1008-16.

Perrone, S., Longini, M., Bellieni, C. V., Centini, G., Kenanidis, A., De Marco, L., ... Buonocore, G. (2007). Early oxidative stress in amniotic fluid of pregnancies with Down syndrome. *Clin Biochem*, *40*(3-4), 177-80.

Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., ... Pasinetti, G. M. (2006). Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *J Biol Chem*, *281*(31), 21745-54.

R Core Team. (2015). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing.

Sauve, A. A. (2008). NAD+ and vitamin B3: from metabolism to therapies. *J Pharmacol Exp Ther*, *324*(3), 883-93.

Slonim, D. K., Koide, K., Johnson, K. L., Tantravahi, U., Cowan, J. M., Jarrah, Z., & Bianchi, D. W. (2009). Functional genomic analysis of amniotic fluid cell-free mRNA suggests that oxidative stress is significant in Down syndrome fetuses. *Proc Natl Acad Sci U S A*, *106*(23), 9425-9.

Soltow, Q. A., Strobel, F. H., Mansfield, K. G., Wachtman, L., Park, Y., & Jones, D. P. (2013). High-performance metabolic profiling with dual chromatography-Fourier-transform mass spectrometry (DC-FTMS) for study of the exposome. *Metabolomics*, *9*(1 Suppl), S132-S143.

Tzoulaki, I., Ebbels, T. M., Valdes, A., Elliott, P., & Ioannidis, J. P. (2014). Design and analysis of metabolomics studies in epidemiologic research: a primer on -omic technologies. *Am J Epidemiol*, *180*(2), 129-39.

Underwood, M. A., Gilbert, W. M., & Sherman, M. P. (2005). Amniotic fluid: not just fetal urine anymore. *J Perinatol*, *25*(5), 341-8.

Uppal, K., Soltow, Q. A., Strobel, F. H., Pittard, W. S., Gernert, K. M., Yu, T., & Jones, D. P. (2013). xMSanalyzer: automated pipeline for improved feature detection and downstream analysis of large-scale, non-targeted metabolomics data. *BMC Bioinformatics*, *14*, 15.

Uppal, K., Walker, D. I., & Jones, D. P. (2016). xMSannotator: an R package for network-based annotation of high-resolution metabolomics data. *Anal Chem*.

Wishart, D. S., Tzur, D., Knox, C., Eisner, R., Guo, A. C., Young, N., ... Querengesser, L. (2007). HMDB: the Human Metabolome Database. *Nucleic Acids Res*, *35*(Database issue), D521-6.

Wold, S., Sjostrom, M., & Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemometr Intell Lab Syst*, *58*(2), 109-130.

Yang, H., Harrington, C. A., Vartanian, K., Coldren, C. D., Hall, R., & Churchill, G. A. (2008). Randomization in laboratory procedure is key to obtaining reproducible microarray results. *PLoS One*, *3*(11), e3724.

Yu, T., Park, Y., Johnson, J. M., & Jones, D. P. (2009). apLCMS – adaptive processing of high-resolution LC/MS data. *Bioinformatics*, *25*(15), 1930-6.

Appendix

I Tables

Table 1. Sample demographic and clinical information.

		Fetal chromosomal constitution		
	All, $n = 119$[a]	Control, $n = 80$	T21, $n = 39$	p-value[b]
Maternal race/ethnicity [n (%)]				0.2785
African American	24 (20.2)	19 (23.8)	5 (12.8)	
Caucasian	74 (62.2)	49 (61.2)	25 (64.1)	
Other	21 (17.6)	12 (15.0)	9 (23.1)	
Fetal gender [n (%)]				0.3013
Female	66 (55.5)	47 (58.8)	19 (48.7)	
Male	66 (55.5)	47 (58.8)	19 (48.7)	
Collection year, MS (median ± IQR)	2007 ± 4.0	2008 ± 5.0	2005 ± 4.5	0.0105
Collection year, AF (median ± IQR)	2007 ± 4.0	2008 ± 4.3	2005 ± 5.0	0.0113
Gestational age, MS [weeks, (mean ± SD)]	17.4 ± 1.6	17.3 ± 1.6	17.6 ± 1.6	0.4478
Gestational age, AF [weeks, (mean ± SD)]	20.5 ± 3.1	20.2 ± 2.9	21.1 ± 3.3	0.1388
Maternal age [years, (mean ± SD)]	30.7 ± 7.7	30.8 ± 7.8	30.4 ± 7.5	0.7661

[a] One sample (S246) with an extreme feature intensity profile was excluded.

[b] Two-sided Pearson's Chi-squared test and t-test performed for nominal and continuous variables, respectively.

Abbreviations: T21, trisomy 21; MS, maternal serum; IQR, interquartile range; AF, amniotic fluid; SD, standard deviation.

Table 2. PLSDA feature selection and predictive performance using SVM classifier.

			10-fold CV BCR		
Sample type	Mode	VIP ≥ 2 (n)	Mean	SD	Permuted mean
Amniotic fluid	C_{18} positive	125	87.31	8.85	50.18
Amniotic fluid	HILIC positive	220	88.58	10.69	50.18
Maternal serum	C_{18} positive	324	78.17	16.24	50.16
Maternal serum	HILIC positive	502	75.37	15.96	50.19

Abbreviations: PLSDA, partial least squares discriminant analysis; SVM, support vector machine; CV, cross-validation; BCR, balanced classification rate; VIP, variable importance in projection; SD, standard deviation.

Table 3. Enriched metabolic pathways with Mummichog p-value < 0.05 and at least four overlap metabolites in amniotic fluid and maternal serum based on 345 and 826 discriminatory features, respectively.

Amniotic fluid			
Pathway	Overlap size	Pathway size	p-value
Vitamin B3 (nicotinate and nicotinamide) metabolism	5	19	0.0010
Butanoate metabolism	5	24	0.0016
Linoleate metabolism	4	18	0.0023
Aspartate and asparagine metabolism	9	66	0.0027
Methionine and cysteine metabolism	7	49	0.0034
Androgen and estrogen biosynthesis and metabolism	8	59	0.0034
Pyrimidine metabolism	6	43	0.0053
Glycosphingolipid metabolism	4	26	0.0086
De novo fatty acid biosynthesis	4	28	0.0119
Fatty acid activation	4	29	0.0140
Glycine, serine, alanine and threonine metabolism	6	52	0.0159
Vitamin E metabolism	4	34	0.0296
Purine metabolism	5	47	0.0345
Arginine and proline metabolism	4	37	0.0445
Maternal serum			
Pathway	Overlap size	Pathway size	p-value
Glycine, serine, alanine and threonine metabolism	16	49	0.0017
Tryptophan metabolism	17	58	0.0027
Alanine and aspartate metabolism	7	19	0.0034
Hexose phosphorylation	6	18	0.0075
Tyrosine metabolism	20	81	0.0094
Arginine and proline metabolism	10	37	0.0107
Porphyrin metabolism	7	25	0.0150
Urea cycle/amino group metabolism	12	49	0.0188
Butanoate metabolism	6	22	0.0230
β-alanine metabolism	4	13	0.0266
Histidine metabolism	5	18	0.0283
Glycerophospholipid metabolism	10	42	0.0295
Aspartate and asparagine metabolism	14	64	0.0480
Lysine metabolism	6	25	0.0494
Drug metabolism - other enzymes	5	20	0.0496

Table 4. Representative discriminatory features in second-trimester T21 amniotic fluid matched to known metabolites by the annotation method indicated.

Metabolite	Adduct	Mode	m/z	RT (s)	VIP	FC
Palmitaldehyde[a]	M+Na[1+]	HILIC	263.24	61	6.05	2.29
Palmitaldehyde[a]	M+Na[1+]	C_{18}	263.24	503	3.72	1.92
Stearidonic acid[a]	M+H[1+]	HILIC	277.21	62	5.62	1.62
Stearidonic acid[a]	M+H[1+]	C_{18}	277.22	416	4.80	0.95
Pregnenolone[a]	M+H+Na[2+]	C_{18}	170.12	181	4.55	0.92
Hexanoylglycine[b]	M+H[1+]	HILIC	174.11	63	4.03	1.04
2-Methoxyestrone 3-glucuronide[a]	M+2H[2+]	HILIC	239.11	61	3.21	-5.31
2-Methoxy-estradiol-17beta 3-glucuronide[a]	M+2H[2+]	HILIC	240.12	93	3.21	5.61
N1N12-Diacetylspermine[c]	M+2H[2+]	HILIC	144.12	305	3.13	0.72
N1N12-Diacetylspermine[c]	M+H[1+]	HILIC	287.24	305	2.92	0.63
N1N12-Diacetylspermine[b]	M+H[1+]	C_{18}	287.24	42	2.73	0.42
N1N12-Diacetylspermine[a]	M+2H[2+]	C_{18}	144.13	41	2.11	0.33
Acetyl-carnitine[d]	M+Na[1+]	HILIC	226.10	129	2.78	3.82
Glutamate[a]	M+H[1+]	C_{18}	148.06	45	2.72	-0.21
Valine[d]	M+H[1+]	HILIC	118.09	196	2.53	-0.30
Estradiol[a]	M+H[1+]	HILIC	273.18	63	2.51	0.79
Uridine[a]	M+Na[1+]	C_{18}	267.06	408	2.49	0.15
Thymine[a]	M+Na[1+]	HILIC	149.03	271	2.49	0.45
Thymine[a]	M+K[1+]	HILIC	165.01	319	2.29	0.40
Acetylspermidine[a]	M+H[1+]	HILIC	188.17	298	2.48	0.65
PC(36:3)[d]	M+H[1+]	C_{18}	784.59	558	2.46	-3.52
alpha-D-Ribose 1-phosphate[a]	M+2H[2+]	HILIC	116.02	393	2.41	0.36
Propionylcarnitine[b]	M+H[1+]	HILIC	218.14	165	2.40	0.54
Arginine[c]	M+H[1+]	C_{18}	175.12	43	2.38	-0.22
Gabapentin[b]	M+H[1+]	C_{18}	172.13	379	2.37	0.21
Taurine[b]	M+H[1+]	C_{18}	126.02	49	2.28	1.82
Taurine[c]	2M+H[1+]	HILIC	251.03	160	2.19	0.48
Taurine[a]	M+Na[1+]	HILIC	148.00	158	2.17	0.40
Serine[b]	M+H[1+]	HILIC	106.05	215	2.28	-0.18
Nandrolone[b]	M+H[1+]	C_{18}	275.20	407	2.22	2.03
3-Mercaptolactic acid[a]	M+K[1+]	HILIC	160.97	208	2.19	0.13
Ornithine[a]	M+Na[1+]	HILIC	155.08	123	2.17	-2.36
Ceramide (d18:116:0)[b]	M+H[1+]	HILIC	538.52	59	2.15	-0.43
Proline betaine[b]	M+H[1+]	C_{18}	144.10	45	2.15	1.98
PC(36:4)[d]	M+H[1+]	C_{18}	782.57	569	2.13	-0.76
Porphobilinogen[b]	M+H[1+]	HILIC	227.10	135	2.13	-0.28
Butyric acid[a]	M+H[1+]	HILIC	89.06	310	2.10	0.48
1-Methylnicotinamide[a]	M[1+]	HILIC	137.07	232	2.04	0.31
Decanoylcarnitine[b]	M+H[1+]	HILIC	316.25	132	2.03	0.41

[a] Putative annotation by Mummichog pathway analysis.

[b] Medium confidence (level 2) annotation by xMSannotator.

[c] High confidence (level 3) annotation by xMSannotator.

[d] Identity confirmed by comparison of m/z and retention time to previously established standards.

Abbreviations: T21, trisomy 21; RT, retention time; VIP, variable importance in projection; FC, fold change; PC, phosphatidylcholine.

Table 5. Mummichog pathway analysis results for enriched glycine, serine, alanine, and threonine metabolism in second-trimester serum of mothers with T21 pregnancies.

m/z	RT (s)	Match name(s)	Adduct	Mode	VIP	FC
106.05	52	Serine[a]	M+H[1+]	C_{18}	3.00	0.61
143.07	329	Choline	M+K[1+]	C_{18}	2.94	-5.35
90.05	241	Sarcosine; alanine	M+H[1+]	HILIC	2.76	0.70
90.05	51	Sarcosine; alanine	M+H[1+]	C_{18}	2.33	0.57
91.06	227	Sarcosine; alanine	M(C13)+H[1+]	HILIC	2.00	0.57
74.06	283	Aminoacetone	M+H[1+]	HILIC	2.61	0.85
88.06	292	Arginine	M+2H[2+]	HILIC	2.59	0.87
175.12	48	Arginine	M+H[1+]	C_{18}	2.27	0.78
176.12	284	Arginine	M(C13)+H[1+]	HILIC	2.45	0.72
132.08	227	Creatine[a]	M+H[1+]	HILIC	2.50	-0.32
132.08	51	Creatine[a]	M+H[1+]	C_{18}	2.47	-0.43
120.06	50	Threonine[a]	M+H[1+]	C_{18}	2.38	0.58
172.04	180	Methionine	M+Na[1+]	HILIC	2.23	-3.41
159.01	426	D-Lactaldehyde	M+HCOOK[1+]	HILIC	2.23	-1.26
76.02	291	Glycolic acid	M[1+]	HILIC	2.22	-4.45
75.03	300	Glycine	M[1+]	HILIC	2.17	-2.89
201.09	117	Ornithine	M+HCOONa[1+]	HILIC	2.16	1.31
119.09	109	Betaine	M(C13)+H[1+]	C_{18}	2.10	0.52

[a] Identity confirmed by comparison of m/z and retention time to previously established standards. Abbreviations: T21, trisomy 21; RT, retention time; VIP, variable importance in projection; FC, fold change.

II Figures

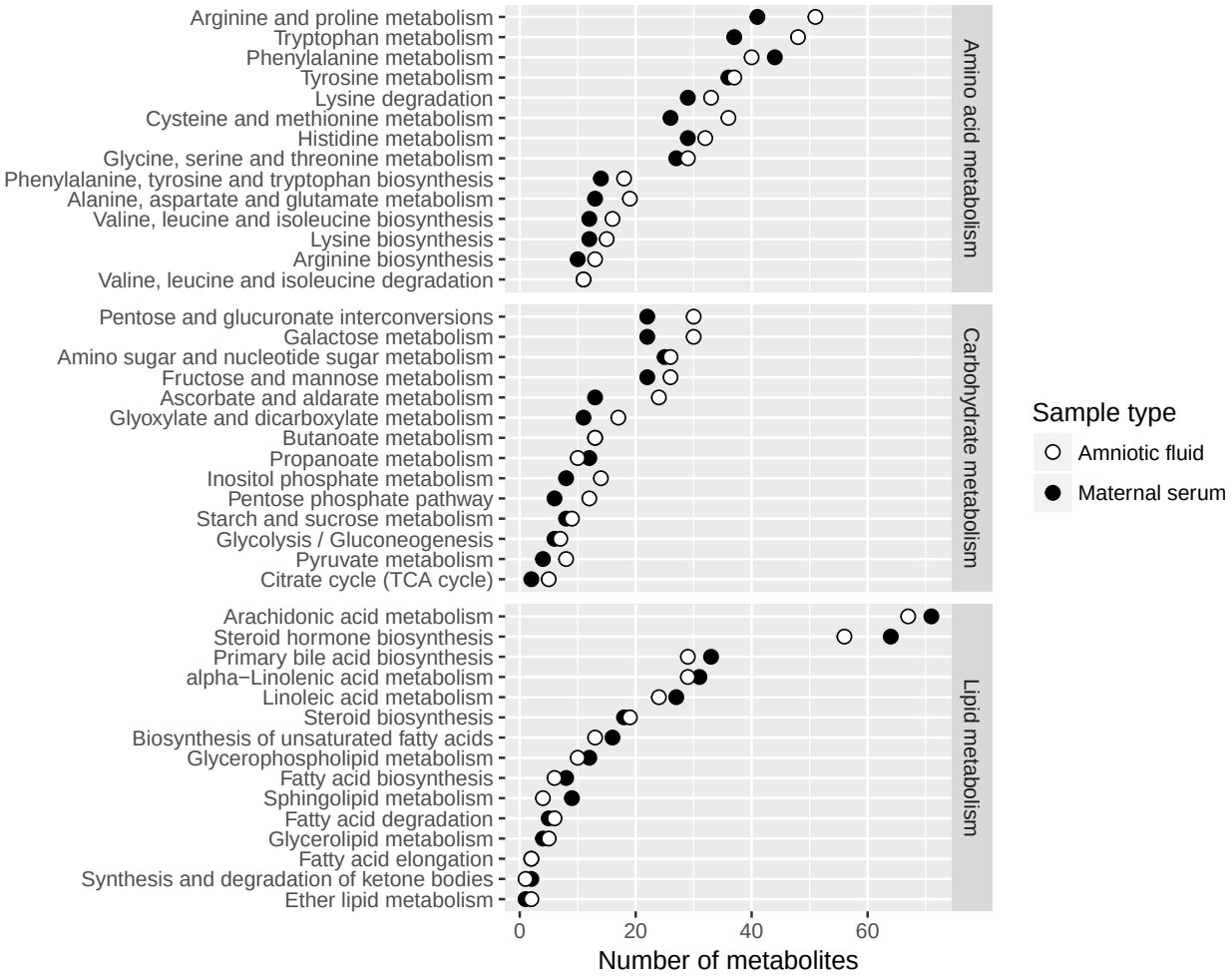

Fig. 1. KEGG mapping of serum and amniotic fluid features detected by HILIC chromatography to pathways of amino acid, carbohydrate, and lipid metabolism. Only features tentatively matched to known KEGG chemicals ($[M+H]^{1+}$, $[M+Na]^{1+}$, 10 ppm mass error) present in human reference pathways were considered. Certain features may be overrepresented if matched to more than one KEGG compound, and compounds may be classified under several metabolic pathways.

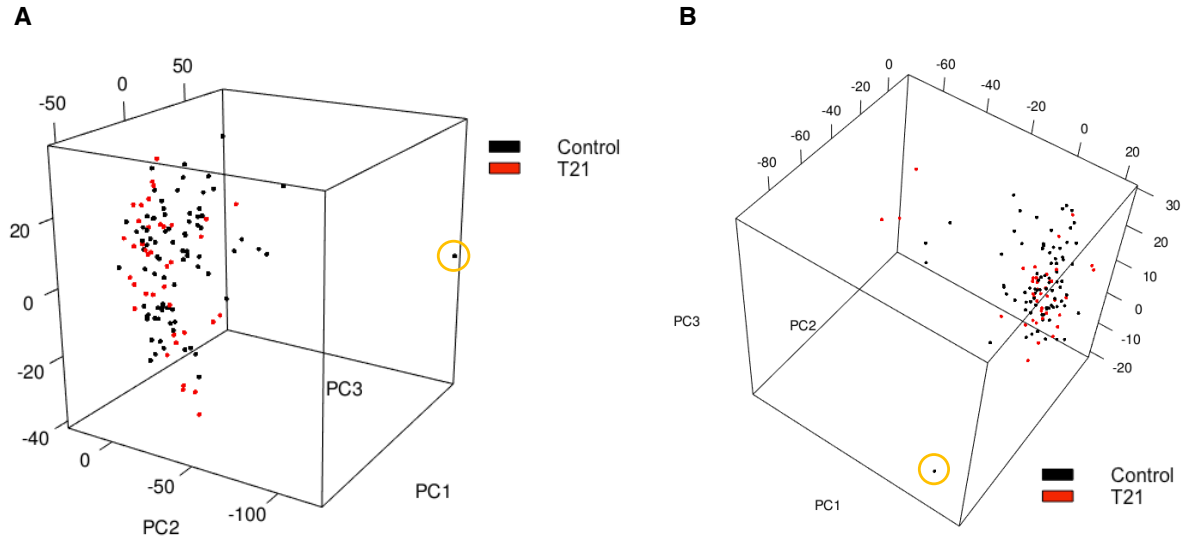

Fig. 2. Score plots of first three principal components from PCA models of amniotic fluid features detected by HILIC chromatography (A) and C_{18} chromatography (B). Control samples ($n = 81$) are shown in black, and T21 samples ($n = 39$) are shown in red. One outlier (S246, circled) was identified in both columns. Abbreviations: PCA, principal component analysis; T21, trisomy 21.

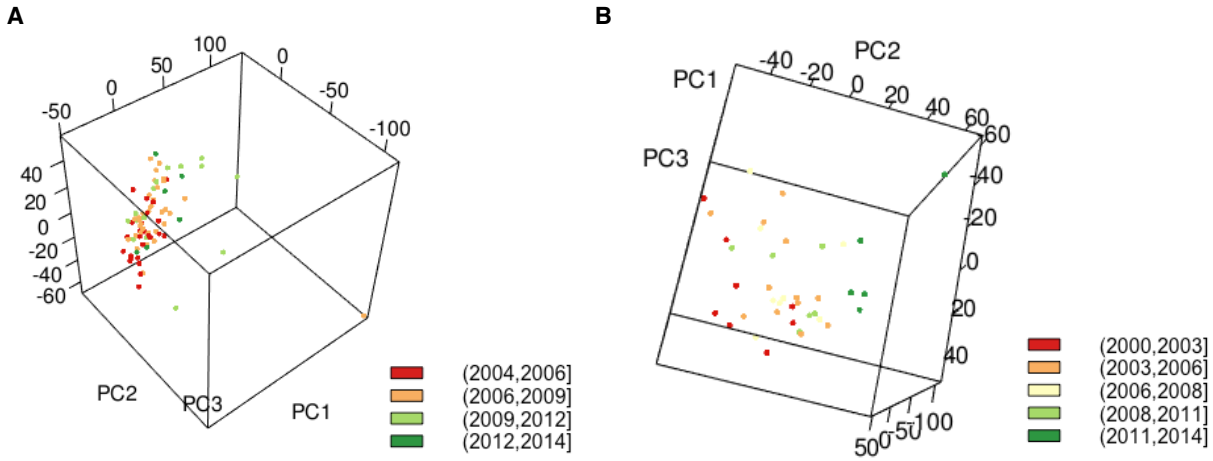

Fig. 3. Score plots of first three principal components from PCA models of amniotic fluid features detected by both chromatography columns in control samples (A) and T21 samples (B). Samples are colored according to year of amniotic fluid collection. Abbreviations: PCA, principal component analysis; T21, trisomy 21.

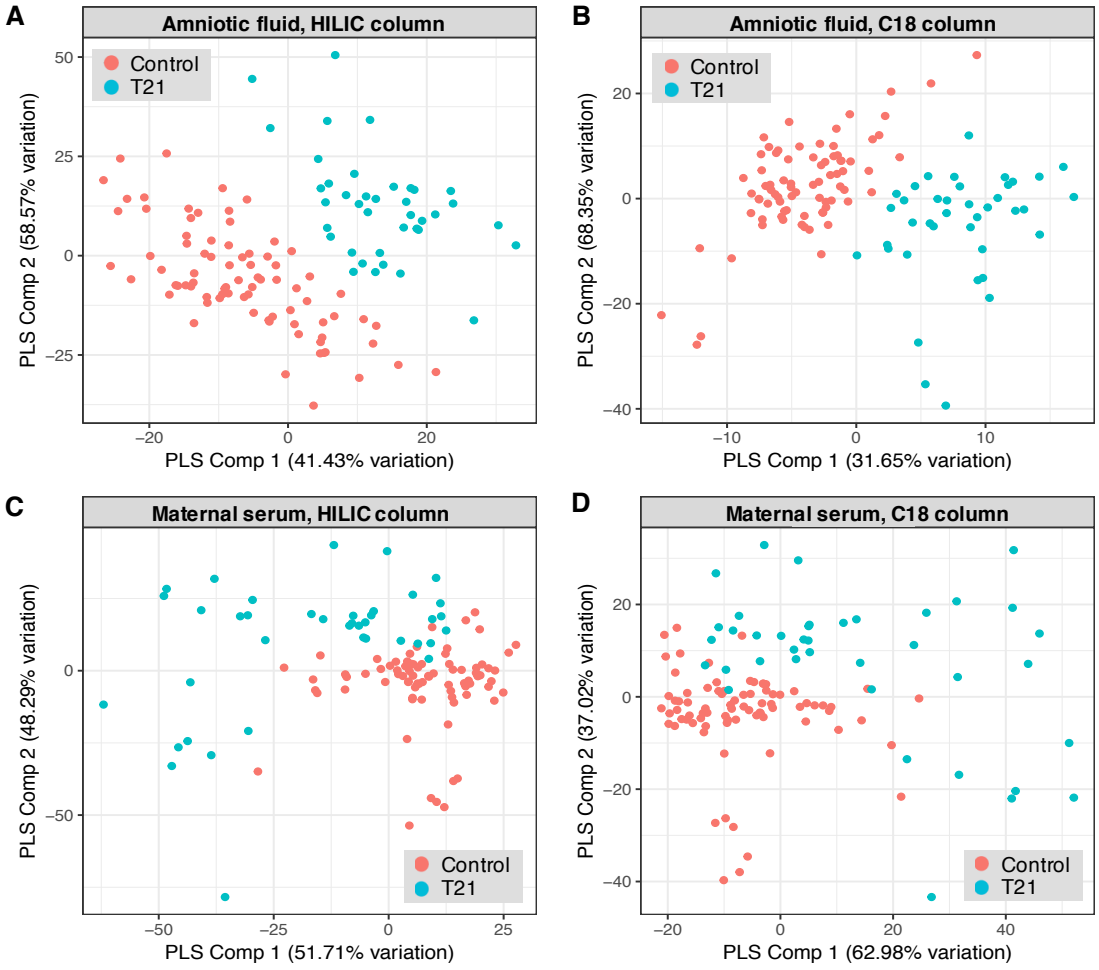

Fig. 4. Score plot of PLSDA model for the HILIC amniotic fluid dataset (A), C_{18} amniotic fluid dataset (B), HILIC maternal serum dataset (C), and C_{18} maternal serum dataset (D). Components are reflective of variation in adjusted feature intensities between sample classes. Abbreviations: PLSDA, partial least squares discriminant analysis; T21, trisomy 21; comp, component.

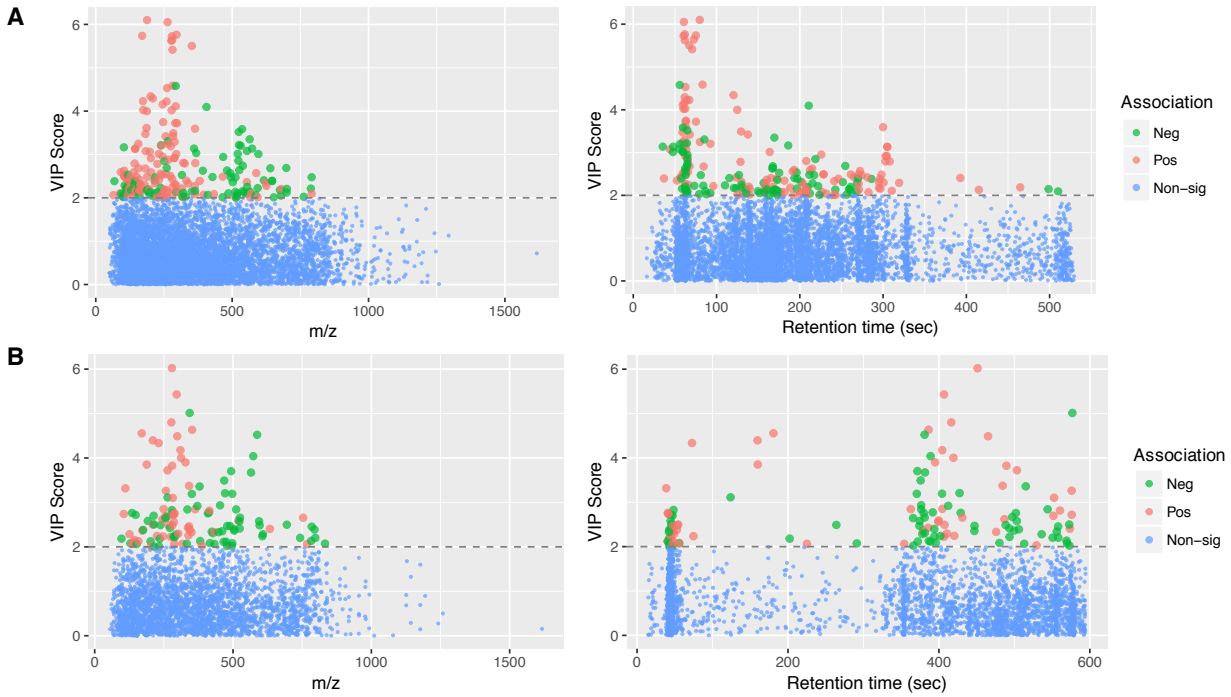

Fig. 5. (A) Manhattan plots of VIP scores from PLSDA amniotic fluid model (HILIC column) as a function of m/z (left) and retention time (right). (B) Corresponding Manhattan plots for C_{18} amniotic fluid features. The dashed line in each panel indicates selection cutoff (VIP $\geq$ 2). Features positively associated with T21 are shown in red; features negatively associated are shown in green. Abbreviations: VIP, variable importance in projection; neg, negative; pos, positive; non-sig, non-significant; PLSDA, partial least squares discriminant analysis; T21, trisomy 21.

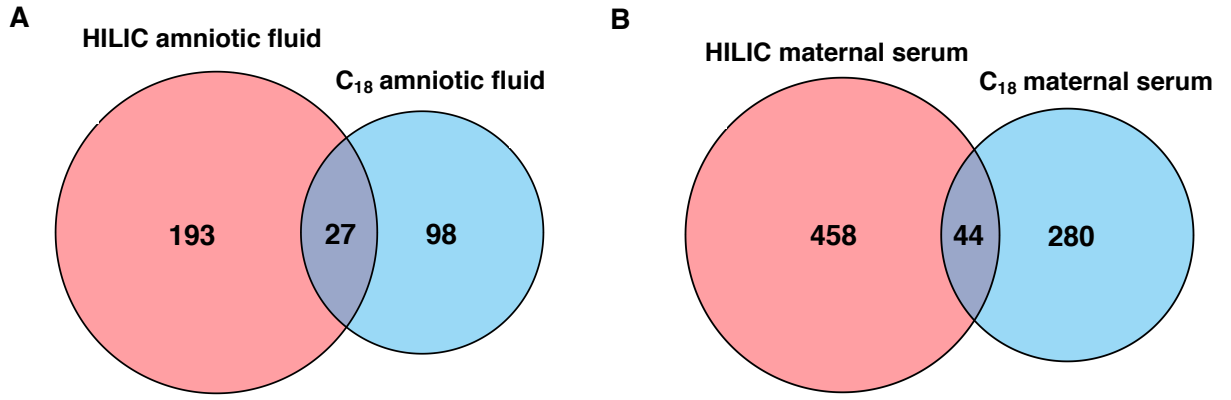

Fig. 6. (A) Overlap of PLSDA-selected discriminatory features (VIP $\geq$ 2) in amniotic fluid across chromatography columns. (B) Overlap of PLSDA-selected discriminatory features (VIP $\geq$ 2) in maternal serum across chromatography columns. Abbreviations: PLSDA, partial least squares discriminant analysis.

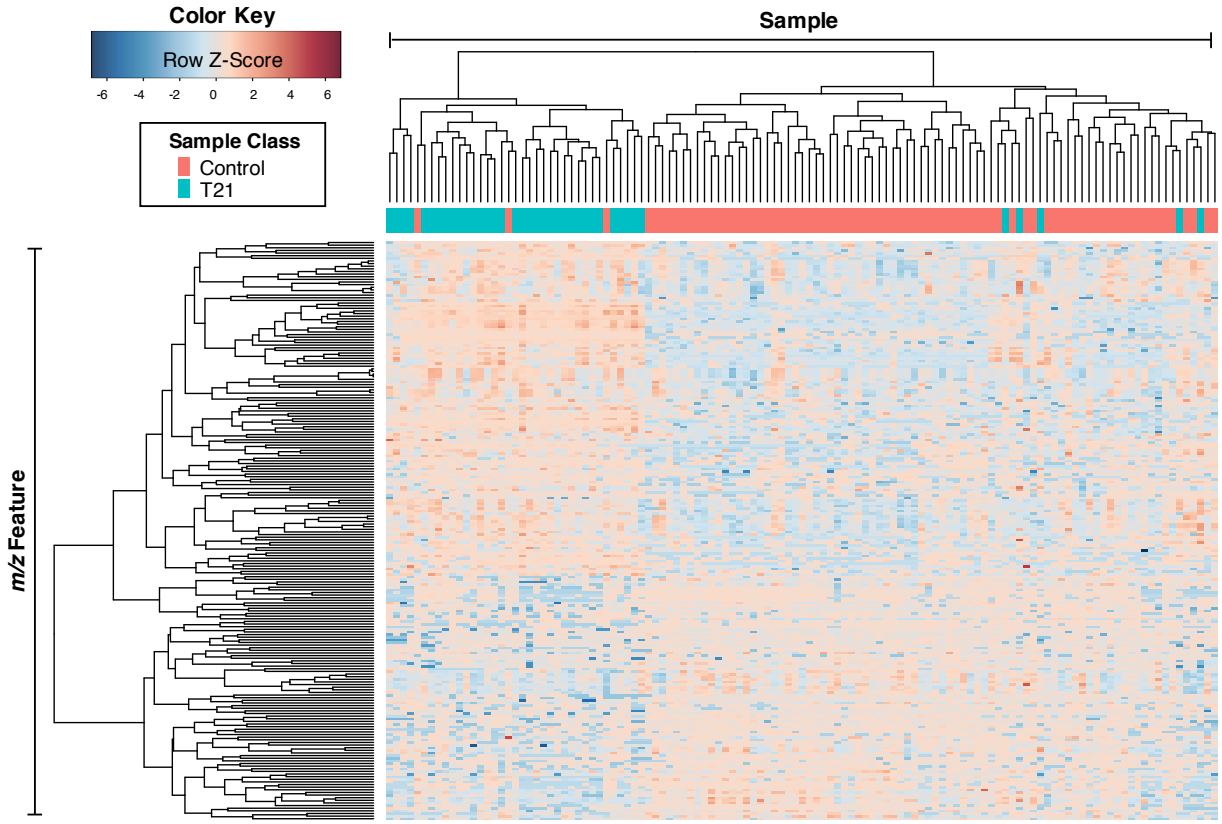

Fig. 7. Heat map from two-way HCA of 220 PLSDA-selected metabolites (VIP $\geq$ 2) detected in amniotic fluid by HILIC chromatography. Clustering of samples is shown on the top axis; clustering of features is shown on the left axis. Cell shading represents row-standardized $\log_2$-transformed feature intensity. Abbreviations: T21, trisomy 21; HCA, hierarchical cluster analysis; PLSDA, partial least squares discriminant analysis; VIP, variable importance in projection.

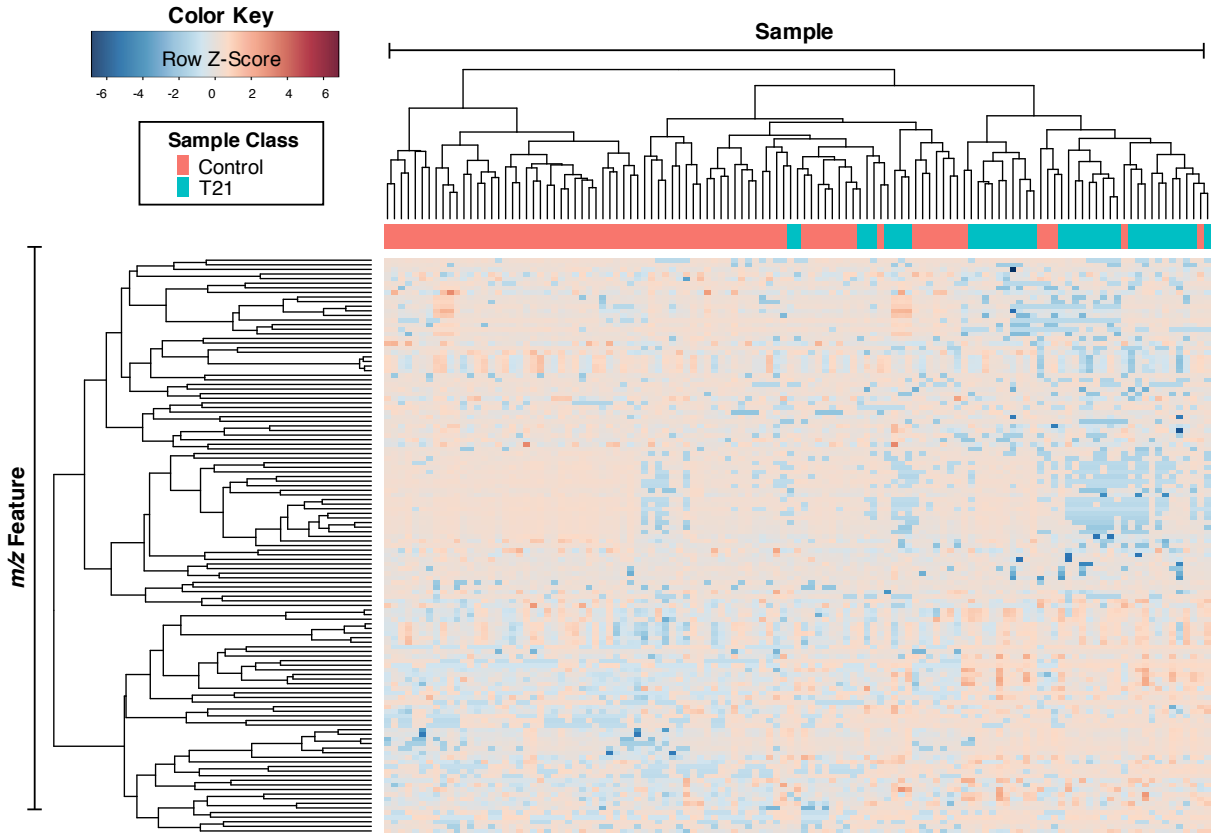

Fig. 8. Heat map from two-way HCA of 125 PLSDA-selected metabolites (VIP $\geq$ 2) detected in amniotic fluid by C_{18} chromatography. Clustering of samples is shown on the top axis; clustering of features is shown on the left axis. Cell shading represents row-standardized $\log_2$-transformed feature intensity. Abbreviations: T21, trisomy 21; HCA, hierarchical cluster analysis; PLSDA, partial least squares discriminant analysis; VIP, variable importance in projection.

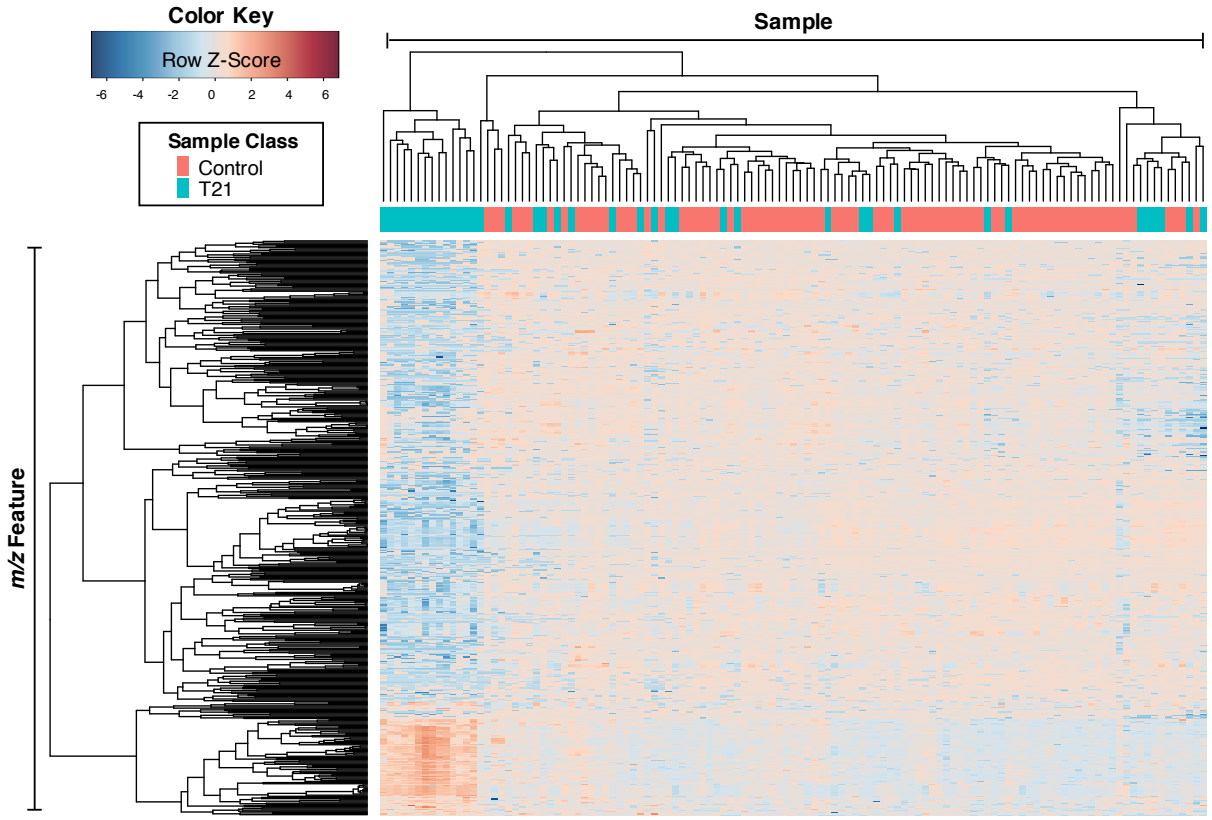

Fig. 9. Heat map from two-way HCA of 502 PLSDA-selected metabolites (VIP $\geq$ 2) detected in maternal serum by HILIC chromatography. Clustering of samples is shown on the top axis; clustering of features is shown on the left axis. Cell shading represents row-standardized $\log_2$-transformed feature intensity. Abbreviations: T21, trisomy 21; HCA, hierarchical cluster analysis; PLSDA, partial least squares discriminant analysis; VIP, variable importance in projection.

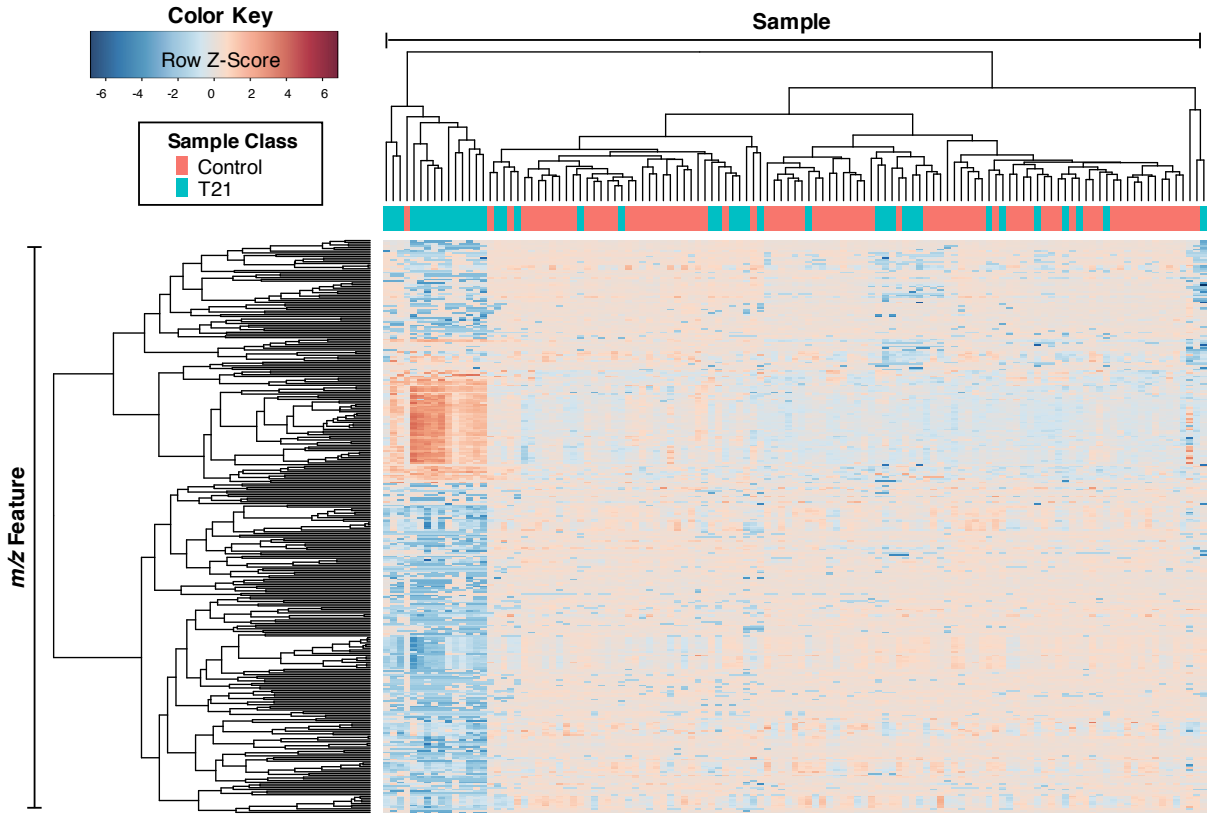

Fig. 10. Heat map from two-way HCA of 324 PLSDA-selected metabolites (VIP $\geq$ 2) detected in maternal serum by C_{18} chromatography. Clustering of samples is shown on the top axis; clustering of features is shown on the left axis. Cell shading represents row-standardized $\log_2$-transformed feature intensity. Abbreviations: T21, trisomy 21; HCA, hierarchical cluster analysis; PLSDA, partial least squares discriminant analysis; VIP, variable importance in projection.