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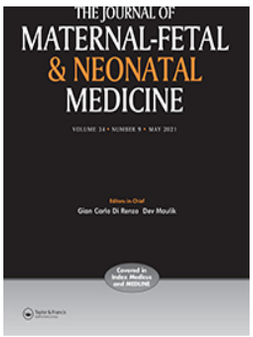

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Metabolomic profiles of mid-trimester amniotic fluid are not associated with subsequent spontaneous preterm delivery or gestational duration at delivery

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ABSTRACT

Introduction: Spontaneous preterm delivery (<37 gestational weeks) has a multifactorial etiology with still incompletely identified pathways. Amniotic fluid is a biofluid with great potential for insights into the feto-maternal milieu. It is rich in metabolites, and metabolic consequences of inflammation is yet researched only to a limited extent. Metabolomic profiling provides opportunities to identify potential biomarkers of inflammatory conditioned pregnancy complications such as spontaneous preterm delivery.

Objective: The aim of this study was to perform metabolomic profiling of amniotic fluid from uncomplicated singleton pregnancies in the mid-trimester to identify potential biomarkers associated with spontaneous preterm delivery and gestational duration at delivery. A secondary aim was to replicate previously reported mid-trimester amniotic fluid metabolic biomarkers of spontaneous preterm delivery in asymptomatic women.

Method: A nested case-control study was performed within a larger cohort study of asymptomatic pregnant women undergoing mid-trimester genetic amniocentesis at 14–19 gestational weeks in Gothenburg, Sweden. Medical records were used to obtain clinical data and delivery outcome variables. Amniotic fluid samples from women with a subsequent spontaneous preterm delivery ($n = 37$) were matched with amniotic fluid samples from women with a subsequent spontaneous delivery at term ($n = 37$). Amniotic fluid samples underwent untargeted metabolomic analyses using liquid chromatography-mass spectrometry. Multivariate random forest analyses were used for data processing. A secondary targeted analysis was performed, aiming to replicate previously reported mid-trimester amniotic fluid metabolic biomarkers in women with a subsequent spontaneous preterm delivery.

Results: Multivariate analysis did not distinguish the samples from women with a subsequent spontaneous preterm delivery from those with a subsequent term delivery. Neither was the metabolic profile associated with gestational duration at delivery. Potential metabolic biomarker candidates were identified from four publications by two different research groups relating mid-trimester amniotic fluid metabolomes to spontaneous PTD, of which fifteen markers were included in the secondary analysis. None of these were replicated.

Conclusions: Metabolomic profiles of early mid-trimester amniotic fluid were not associated with spontaneous preterm delivery or gestational duration at delivery in this cohort.

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Supplemental data for this article can be accessed here.

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Introduction

The spontaneous onset of labor, both at term (delivery at or after 37 weeks of gestation) and at preterm (delivery before 37 weeks of gestation), is multifactorial, complex and not completely understood. Several mechanistic factors initiate maternal-fetal inflammatory processes [1–3] that stimulate the production and expression of cytokines and cytotoxic molecules with subsequent uterine activity [4]. This inflammation can alter the local and systemic metabolic profile [5]. Examining interactions between inflammation and metabolic changes during gestation using metabolomics (comprehensive measurement of small molecule metabolites in a biological sample) may thereby increase our understanding of the etiology of spontaneous preterm delivery (PTD), and gestational duration at delivery [5]. It is also a promising method for biomarker discovery [6,7], where especially amniotic fluid, rich in metabolites, becomes a highly interesting biological matrix [5].

The number of studies using metabolomics analyses for biomarker research has increased substantially during the last years. A few studies have used metabolomics to identify biomarkers of spontaneous PTD in amniotic fluid from symptomatic women, with either spontanteous preterm labor with intact membranes (PTL) or preterm prelabor rupture of membranes (PPROM), in the late second or third trimester [8,9]. They found that higher levels of amino acids, unsaturated hydroxyl fatty acids and fatty aldehydes [8], i.e. products of the intermediate metabolism of mammalian cells and xenobiotic compounds, were associated with spontaneous PTD [9]. However, only a limited number of studies have performed metabolic profiling of mid-trimester amniotic fluid in relation to spontaneous PTD in asymptomatic women [7,10–12], of which the majority include a small number of subjects and a very broad sampling window. The identification of early biomarkers in yet asymptomatic women may be of greater value since these could predict women at risk of spontaneous PTD.

The aim of this study was to investigate metabolomic profiles of mid-trimester amniotic fluid obtained from asymptomatic women and to examine associations with a subsequent spontaneous PTD and gestational duration at delivery. Second, the study aimed to replicate previously reported mid-trimester amniotic fluid metabolite biomarkers associated with spontaneous PTD.

Material and methods

Inclusion and exclusion criteria

Women ≥18 years of age with a singleton pregnancy, intact membranes and without signs of infection who underwent a mid-trimester genetic amniocentesis at Sahlgrenska University Hospital/Östra, Gothenburg, Sweden were recruited to a large prospective pregnancy cohort study. Women enrolled between September 2008 and July 2017 were selected for this nested case-control study. Amniocentesis was performed between 14 and 19 weeks of gestation in line with clear clinical indications: advanced maternal age (≥35 years), first trimester combined screening indicating a high risk of chromosomal abnormality, family history of chromosomal abnormalities or genetic diseases, or anxiety. Women who had multiple pregnancy, infection with human immunodeficiency virus, hepatitis B or C virus and women with known or suspected fetal abnormalities were considered ineligible as well as women undergoing amniocentesis during times when study samples could not be collected. Women who could not provide an informed consent in Swedish, who declined participation, who had an initial twin gestation with vanishing twin, or where an insufficient amount of amniotic fluid was collected were excluded.

Selection of cases and controls

Medical records were used to obtain subjects' demographics and clinical data. Women with a subsequent spontaneous PTD (cases) were matched to women with a subsequent delivery at term (controls) on a 1:1 ratio. The group of women with a spontaneous delivery at term was limited to gestational weeks 38 + 0–41 + 6 to achieve a more homogeneous group. Matching was performed according to the following criteria; (1) gestational age at sampling (±3 days), (2) parity (nulliparous/multiparous), (3) *in vitro* fertilization (IVF; yes/no), (4) maternal age at sampling (±2 years), (5) body mass index (BMI) group (according to the World Health Organization (WHO) definitions of BMI categories: underweight (<18.5 kg/m^2); normal weight (18.5–24.9 kg/m^2); pre-obesity (25.0–29.9 kg/m^2); obesity class I (30.0–34.9 kg/m^2) and obesity class II (35.0–39.9 kg/m^2)), and (6) pregnancy complications or maternal chronic diseases. Women who fulfilled the above-described criteria and who had the closest sampling dates to the cases were selected as controls. Matching was successful with a few exceptions where e.g. the criteria of BMI category could not be matched

and where the matching was instead based on the actual BMI value.

Collection and processing of samples

Amniotic fluid samples for research (approximately 3 ml) were collected during the clinical mid-trimester genetic amniocentesis. The procedure was ultrasound-guided, transabdominal, and performed using a 22-gauge needle. Immediately after withdrawal, samples were stored at 4–8 °C for 4 ± 1.9 h (mean ± SD) before centrifugation for 20 min at 12,000 *g*, 4 °C. Supernatants and pellets were thereafter separated, aliquoted, frozen and stored at −80 °C awaiting analysis. Parts of the larger prospective pregnancy cohort study have previously been used for a few different subset analyses [13–15].

Ethical approval

The study was approved by the Ethics Review Board at the University of Gothenburg, Sweden (Dnr. Ö 639-03, T 318–08, T 694-11, 2019-06022). All participants had given their written informed consent.

Untargeted LC-MS metabolomics

Amniotic fluid samples were analyzed by untargeted UHPLC-QTOF metabolomics at the Chalmers Mass-Spectometry Infrastructure at Chalmers University of Technology. In brief, samples were thawed at 4 °C and a 30 µL aliquot was mixed with 200 µL cold (4 °C) acetonitrile on a 96 well plate. The plate was sealed, mixed using an orbital shaker and centrifuged for 10 min at 500 g (4 °C). The remaining supernatants were then filtered through 0.2 µm Captiva ND plates (Agilent Technologies) onto a 96 well plate.

The samples were analyzed using both reverse phase and HILIC chromatography on an Agilent 1290 Binary LC system coupled to an Agilent 6550 quadrupole time-of-flight mass spectrometer with electrospray ionization operated both in positive and negative ionization. For reverse phase, sample extracts were kept at 4 °C and injected onto a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm), maintained at 45 °C. Mobile phases were ultrapure water (A) and LC-MS grade methanol, both containing 0.04% formic acid, and a linear gradient from 5% to 100% B over 6 min were used for sample elution. Flowrate was set to 0.4 ml/min. For HILIC chromatography, a Waters Acquity UPLC BEH Amide NH_2 column (100 × 2.1 mm, 1.7 µm), maintained at 45 °C, was used and mobile phases were water (A) and 90/10 acetonitrile/water (B), both with 10 mM ammonium formate at pH 3.5. The gradient started at 100% B where held for 1 min, ramped to 70% B over 7 min and returned to initial conditions after 0.1 min. Flowrate was set to 0.4 ml/min. Mass spectra were acquired across a mass range of 50–1600 *m/z* at 1.67 spectra/second. The capillary voltage was set at 3500 V. The source parameters were set with gas temperature at 175 °C, flow at 12 L/min, nebulizer at 45 psig, sheath gas at 350 °C and sheat gas flow at 11 L/min. MS data were acquired with Mass Hunter Workstation Data Acquisition (Agilent Technologies).

Pre-processing pipeline

Raw centroid instrument files were converted to .mzML file format using the *MSConvertGUI* software [16] before being imported into the R v3.5.1 open source environment [17]. The XCMS v3.4.4 package was used for peak picking, retention time alignment, grouping and filling of missing features [18]. XCMS parameters were optimized using a combination of the IPO v1.8.1 package [19] and manual optimization performed on all QC samples spanning the entire injection sequence (Table S1). Imputation of values still missing after XCMS peak filling was performed using an in-house RandomForest-based algorithm (https://gitlab.com/CarlBrunius/StatTools; mvImpWrap() function). The obtained data were corrected for within- and between-batch intensity drift using the *batchCorr* package [20], after which features with high variability among QC samples (RSD > 30%) were filtered out.

Finally, grouping of features (isotopes, adducts and fragments) corresponding to the same metabolites was achieved using the RAMClustR package [21]. The similarity parameters (σ_t, σ_r) were optimized using an in-house procedure. The number of features per LC-MS mode at key steps of the pre-processing pipeline is reported in Table S1. R scripts for parameter optimization and pre-processing are available from the authors upon request.

Targeted LC-MS metabolomics

In the secondary analysis, targeted analyses were performed on metabolite biomarkers previously reported in mid-trimester amniotic fluid of asymptomatic women, as being associated with the subsequent development of spontaneous PTD. Metabolite biomarkers in amniotic fluid of symptomatic women (PTL or PPROM, with or without signs of inflammation or

infection) in the late mid-trimester were not included due to the high likelihood that these would reflect different biological mechanisms.

Investigation of the reported biomarker candidates from the literature was performed in the collected data by extracting features corresponding to a shortlist of adducts for the suggested neutral masses ($[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M+CH_3OH+H]^+$, $[M+ACN+H]^+$ and $[M+2H]^{2+}$ in positive mode and $[M-H]^-$, $[M-H_2O-H]^-$, $[M+Na-2H]^-$, $[M+K-2H]^-$, $[M+Cl]^-$, $[M+FA-H]^-$, $[M+HAc-H]^-$ and $[M-2H]^{2-}$ in negative mode) within a mass difference of 10 ppm. Significance of association was assessed using paired *t*-tests between case/control pairs. Features not matching the suggested candidates were filtered out, first by MS (if e.g. matching to an isotope instead of main fragment) and later by MSMS by comparing fragmentation patterns to HMDB hits of the suggested biomarker candidates.

Statistical analysis

Differences in baseline characteristics between cases and controls were analyzed by Mann–Whitney *U* test and presented as median (interquartile range (IQR)) for continuous variables while categorical data were analyzed by Chi-square test or Fishers exact test (when below five individuals at any level) and presented as numbers [frequency distribution (%)], using SPSS 24.0 and 25.0 for Windows XP (SPSS Inc., USA). Differences were considered statistically significant at $p < .05$ using a two-sided alternative hypothesis.

To establish that the metabolomics data was fit for purpose (sanity check), we first investigated the association between metabolome and gestational age at sampling (not related to the study aims). In the primary analysis, we investigated the associations of the mid-trimester amniotic fluid metabolome with both spontaneous PTD and gestational duration at delivery. Multivariate analysis for both sanity check and primary analysis was performed using random forest with unbiased variable selection within repeated double cross-validation, using the MUVR R package v 0.0.973 [22]. Analysis of spontaneous PTD-vs-control was investigated as case/control pair-dependent multilevel analyses using log fold change between cases and controls as independent variables [22–24]. Random forest regression models using MUVR were performed for the association of the amniotic fluid metabolome with gestational age at sampling and gestational duration at delivery. Statistical significance of multivariate models was assessed using permutation tests [25] and considered significant at one-sided $p < .05$ (i.e. actual model performing better than permuted null hypothesis distribution).

Results

Characteristics of the study population

During the study period, 3128 women underwent genetic amniocentesis. Of these, 1218 women (38.9%) were enrolled, 762 women (24.4%) were ineligible and 1148 women (36.7%) were excluded, the majority of which declined participation. Of the 1218 enrolled women, 28 had a termination of pregnancy due to chromosomal abnormalities, 7 were lost to follow-up, 3 had a miscarriage and 16 were incorrectly enrolled (blood borne viral infection, suspected fetal abnormalities or initial twin gestation with fetal demise of one twin or with vanishing twin). These were excluded, leaving 1164 women to constitute the selection group for cases and controls. PTD occurred in 6.4% of the women (75/1164), of which 68.0% (51/75) were spontaneous PTD, including women with PTL ($n = 25$) and women with PPROM ($n = 26$). Further, women with medically indicated onset of labor, both at term and preterm ($n = 294$), were excluded, leaving 870 women with a spontaneous onset of labor at term or preterm for the selection of spontaneous cases and controls. Protocol deviations in process handling or sampling deviations (discolored amniotic fluid due to blood contamination) occurred in a few cases ($n = 4$) and these were thus excluded. Women with severe chronic diseases or conditions that could potentially contribute to the occurrence of spontaneous PTD or affect the metabolome, such as uterus malformations, polycystic ovary syndrome, diabetes mellitus, gestational diabetes or malignity ($n = 6$), and women with confirmed or suspected maternal or fetal genetic abnormalities ($n = 4$) were also excluded, leaving 37 women with a spontaneous PTD. These were matched to an equal number of controls ($n = 37$). One case sample was lost during the analysis due to instrument malfunction, leaving 36 matched case/control pairs ($n = 72$) for the statistical analyses (Figure S1).

Maternal and neonatal characteristics of the 36 cases and matched controls are presented in Table 1. There were no significant differences between the groups except the obvious difference in gestational duration at delivery and neonatal birth weight.

Table 1. Characteristics of cases with spontaneous PTD and matched controls.

Variable	Spontaneous PTD ($n = 36$)	Controls ($n = 36$)	p
Gestational duration at delivery (weeks + days)	36 + 1 (33 + 5–36 + 4)	39 + 6 (38 + 6–40 + 5)	
Birth weight (grams)	2643 (2199–3018)	3587 (3280–3974)	
Matching variables			
Gestational age at sampling (weeks + days)	15 + 5 (15 + 0–16 + 1)	15 + 4 (15 + 2–16 + 0)	.92
Nulliparous	13 (36.1%)	12 (33.3%)	.80
IVF	3 (8.3%)	3 (8.3%)	1.00
Maternal age at sampling (years)	37 (35–39)	37 (35–39)	.74
Maternal BMI at first prenatal visit	25.6 (22.7–28.1)	25.8 (22.2–27.7)	.99
Other variables			
Smoking at first prenatal visit	3 (8.3%)	0 (0.0%)	.24
Previous PTD	5 (13.9%)	5 (13.9%)	1.00
Mode of delivery			
Vaginal delivery	27 (75.0%)	30 (83.3%)	.38
Vacuum extraction or forceps	1 (2.8%)	3 (8.3%)	.61
Cesarean section	8 (22.2%)	3 (8.3%)	.19
Neonatal sex			.64
Male	15 (41.7%)	17 (47.2%)	
Female	21 (58.3%)	19 (52.8%)	
Apgar score <7 at 5 min	1 (2.8%)	0 (0%)	1.00

Continuous variables were analyzed using a Mann-Whitney *U* Test and are presented as the median (IQR). Categorical variables were analyzed using Pearson Chi-Square or Fisher's Exact Test (when the expected values in any of the cells are below five individuals) and are shown as *N* (%). Bold text indicate statistical significance at $p < .05$ using a two-sided alternative hypothesis.

Primary analysis by untargeted metabolic profiling

Data integrity was assessed by regressing the gestational age at sampling on the measured amniotic fluid metabolome (the model's predictive ability (Q2) = 0.22, $p = .00018$). There was no association in the multilevel random forest analysis between the metabolomic profile of mid-trimester amniotic fluid and spontaneous PTD (classification rate (CR) 51%). Nor, any associations were found in regards to gestational duration at delivery (Q2 = −0.03).

Secondary analysis by targeted metabolic profiling

Four studies by two different research groups relating mid-trimester metabolomes to spontaneous PTD were identified [7,10–12], providing 32 potential biomarker candiates. However, *m/z* values of all the ten metabolites identified by Graça et al. [7] and seven of the metabolites identified by Virgiliou at el. [12] were either metabolite fragments, metabolite adducts, newly identified metabolite variations or unknown features and reported with only two decimals resolution. They were thus unsuitable for matching to exact mass and were consequently not included in the secondary analysis, leaving three studies and 15 metabolites for replication using targeted metabolomics (Table 2). None of these metabolites were associated with spontaneous PTD in our dataset at nominal $p < .05$.

Discussion

This study aimed to identify potential biomarkers for spontaneous PTD, using metabolomic profiling of early mid-trimester amniotic fluid from asymptomatic women. It further aimed at investigating the association between the early amniotic fluid metabolomic profiling and gestational duration at delivery. We employed advanced multivariate prediction models with reduced risk of overfitting [22,26]. The metabolomic profile did not associate with either spontaneous PTD or gestational duration at delivery, indicating that biological signals in amniotic fluid collected at early mid-trimester may be of insufficient strength for accurate risk predictions of spontaneous PTD and gestational duration at delivery at the individual level. The metabolomic profile did, however, correlate to gestational age at sampling, indicating that the amniotic fluid, at least partly, reflect the biological process of pregnancy development. Such "pregnancy clocks" have previously been reported using different omics techniques [27–31]. In the secondary analysis, we aimed to replicate previously reported amniotic fluid metabolites associated with spontaneous PTD in asymptomatic women with singleton pregnancies in the mid-trimester. None of the 15 potential metabolic candidate biomarkers from previous studies [10–12] were associated with spontaneous PTD in our dataset.

Spontaneous PTD is a complex, heterogenous condition where several pathophysiological mechanisms, such as inflammation, infection, oxidative stress and placental dysfunction, have been proposed. Previous studies using metabolomics on mid-trimester amniotic

Table 2. Metabolites included in the secondary analysis.

Metabolite	HMDB identifier	Monoisotopic mass[a]	Regulation in spontaneous PTD (effect size cases vs controls)	Publication	*p*	Commentary
Alanine	161	89.05	Decrease (−7%)	[10]	.003	NS
Alanine	161	89.05	N/A	[11]	N/A	NS
Alfa-oxoisovalerate	0019	116.05	Decrease	[11]	N/A	NS
Allantoin	0462	158.04	Increase (47%)	[10]	.002	NS
Citrate	0094	192.03	Decrease (−6%)	[10]	0.005	NS
Citrate	0094	192.03	N/A	[11]	N/A	NS
Glucose	0122	180.06	Decrease	[11]	N/A	NS
Glutamic acid	014	147.05	Increase	[12]	N/A	NS
Histidine	0177	155.07	N/A	[11]	N/A	NS
Inositol/myo-inositol	0211	180.06	Decrease (−7%)	[10,12]	.002	NS
Inositol	0211	180.06	Decrease	[12]	.002	NS
Myo-inositol	0211	180.06	Decrease (−7%)	[10]	.002	NS
Isoleucine/leucine	13,773	N/A	Decrease	[11]	N/A	NS
Lysine	3405	146.11	Decrease	[11]	N/A	NS
Phenylalanine	159	165.08	N/A	[11]	N/A	NS
Proline	162	115.06	Decrease	[11]	N/A	NS
Pyruvat	243	88.02	Decrease	[12]	N/A	NS
Tyrosine	158	181.07	Decrease	[11]	N/A	NS
Valine	N/A	117.08	N/A	[11]	N/A	NS

[a]Monoisotopic mass for annotated metabolites from HMDB database.
Commentary reflects the findings in our dataset. NS: not significant; N/A: information not available.

fluid [7,10] have found decreased levels of several amino acids in spontaneous PTD cases. As amino acids are transported between the maternal and fetal circulation by the placenta, their findings corroborate that disturbances in placental function may play a role in the spontaneous PTD etiology [32]. Increased levels of allantoin, a metabolic intermediate produced from uric acid, previously described as a potential marker of oxidative stress, has also been found in spontaneous PTD cases [10].

Apart from the studies on asymptomatic women [7,10–12], there are also studies describing the metabolomic profiles of term and preterm cases sampled when labor has already commenced. Menon et al. [33] identified several altered amniotic fluid metabolites in spontaneous PTD, a majority of them linked to liver function and fatty acid metabolism. Fatty acids were also found to differ between study groups in the study by Lizewska et al. [34], not between the term and preterm group, but between women who delivered preterm and women with threatening preterm labor who went on to deliver at term. Romero et al. [9] report an association between the presence of intra-amniotic inflammation (IAI) and an altered amniotic fluid metabolite composition. Carbohydrates (mannose, galactose and fructose) were relatively increased in patients with PTL who delivered at term while amniotic fluid amino acids (alanine, glutamine and glutamic acid) were decreased, with the opposite state in patients with PTL and IAI.

Comparing results from metabolomics studies of symptomatic women with results from studies of asymptomatic women is problematic for several reasons. First, the origin and composition of amniotic fluid changes with advancing gestational age [35]. Second, metabolic adaptions to support the growing fetus occur during pregnancy. Early gestation is characterized by an anabolic state, where lipids are stored and insulin sensitivity increased, compared to late gestation which should rather be considered a catabolic phase [36]. Third, amniotic fluid samples from women with threatening PTD represent cases where the condition is already in its most advanced stagesand may, therefore, express other markers than those present in the early stages.

Spontaneous PTD has been heavily linked to inflammation, both infection-mediated and sterile, and elevated levels of prostaglandins, key mediators in the inflammatory response, have been demonstrated in amniotic fluid in women with PPROM, regardless the presence of infection [37]. Prostaglandins are derived from the fatty acid arachidonic acid. In the presence of inflammation leading to elevated concentrations of prostaglandins, one would expect to find altered levels of its fatty acid precursor. However, in our cohort, the majority of women had late PTD where infection and inflammation are less frequently involved [38], making it less likely to find such patterns.

Several factors may contribute to the discrepancy between our study and previous studies on asymptomatic women [7,10–12]. The sampling of previous studies occurred within a broader gestational age interval compared to our study which rather reflects early mid-trimester. Metabolic changes appear during

pregnancy [39], which might partly explain previous findings being different from ours since their samling took place up to six weeks later in pregnancy. In all studies of Graça et al. [7,10,11], the number of samples were limited ($n = 11–14$) and women were of a broad age range (13–42 years). Our sample size was threefold larger and more homogenous in regards to gestational age at sampling, as previously mentioned, but also in regard to maternal age. It is known that maternal age and lifestyle factors may affect metabolic profiles and it is possible that our cohort and those of previous studies may be of significant biological difference. In a systematic review from 2019 using metabolomics to identify pathways and biomarkers of PTD [40], Carter et al. concluded that there is an inconsistency in study design and methodology in regard to biological samples, definition of the outcome, confounding factors and covariates, and metabolite identification. Highly heterogenous studies, together with potential internal and external factors, unknown to us at this time, can further contribute to the discrepancy.

Several of the previously reported metabolites associated with spontaneous PTD were related to energy and amino acid metabolism [7,12]. Many of these are affected by sample management such as pre-centrifugation temperature and time [41]. It is, therefore, possible that the variability in pre-centrifugation time in the present study could have obfuscated true underlying associations of spontaneous PTD with these metabolites or, alternatively, that the previous reporting of these metabolite candiates may in fact be artefactual. Although we cannot arbiter between these potential options, we have reason to believe that the data integrity in our study is fundamentally sound, from the sanity check of being able to model gestational age at sampling. In the study of Graça et al. [7], samples were centrifuged after sampling, as in our study, but using slightly different conditions. In the study of Virgiliou et al. [12], samples were frozen immediately after collection and later thawed and processed. There was, however, no information about preparation time or storage temperature during preparation. Further, the results from Graça et al. [10] revealed only small metabolite changes in a very limited sample size. In a study from the same group in 2013 [11], mid-infrared spectroscopy was used, a technique that report chemical classes rather than individual metabolites. Results should, therefore, be interpreted with caution, as the authors also state.

The major strengths of this study are the unique cohort of mid-trimester amniotic fluid samples from asymptomatic women, a very low proportion of lost to follow-up, an extensive database with clinical variables and thorough selection criteria for cases and controls. Another strength is the use of a validated and established pipeline for comprehensive coverage of the metabolomic profile using untargeted metabolomics. Finally, we employed a robust data processing pipeline using a random forest implementation designed to minimize overfitting and false positives.

Potential limitations are that the spontaneous PTD group consisted of women with both PTL and PPROM, where the respective phenotypes may differ. The prevalence of PTD in Sweden is only 5.6% [42] and despite a relatively large cohort, the sample size did not allow differentiation. Further, women undergoing genetic amniocentesis are often of a more advanced maternal age and have a higher risk of fetal chromosomal abnormalities than the overall population, decreasing generality. However, due to the risks with such invasive sampling, mid-trimester amniotic fluid samples for research can only be performed in conjunction with a clinical procedure. A criterion for enrollment was that the women understood the written and oral information about the study, provided in Swedish. Enabling women with other languages to participate could potentially have given us other results since it, most likely, would have increased our population size, and since it is recognized that both the spontaneous PTD rate and the metabolome differs between ethnicities. Another important limitation is the diversity in sample preparation time which may have affected the ability to identify potentially important but not as stable markers. Further, the study design using matched cases and controls was not optimal from a gestational duration at delivery perspective, limiting the likelihood of discovering associations for such a continuous variable. Finally, sampling only occurred once during gestation and within a very limited time frame of pregnancy (14–19 gestational weeks). Sampling at later gestational ages might have led to other results, however, this can only be speculated on. This is neither anything that we can influence in a cohort of asymptomatic women like this one, as such samples are scarce and collected in accordance with clinical routine where amniocentesis is used very restrictively in Sweden.

Conclusions

Our results do not provide evidence that the metabolic profile in the early mid-trimester amniotic fluid is associated with either spontaneous PTD or gestational duration at delivery. Possibly, biological signals in early

mid-trimester amniotic fluid may be of insufficient strength for accurate risk predictions of spontaneous PTD and gestational duration at delivery at the individual level.

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Disclosure statement

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