

Amniotic fluid metabolic fingerprinting indicated metabolites which may play a role in the pathogenesis of foetal Down syndrome — a preliminary report

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ABSTRACT

Objectives: Down syndrome is the most common human chromosomal aberration. It is commonly known that it is a genetic-based disease, but still, pathomechanisms which lead to observed disorders have not been explained. The objective of this study was to determine the metabolic fingerprinting of the amniotic fluid women carrying foetuses with Down syndrome (DS).

Material and methods: The study and control groups consisted of women who underwent routine amniocentesis between the 15th and 18th week of gestation. After analysis of the karyotyping results, 13 women with foetal DS were chosen. For the control group, 13 healthy patients with uncomplicated pregnancies who delivered healthy newborns at term was selected. Amniotic fluid was analyzed using liquid chromatography combined with high resolution mass spectrometry.

Results: In the amniotic fluid of women with foetal DS compared to patients with healthy foetuses, we reported significant differences in the level of four metabolites: methylhistidine, hexanoylcarnitine, diacetylspermine and p-cresol sulfate which may be connected with improper development of nervous system and muscles. We detected bacterial metabolite, which support the latest thesis about non-sterile intrauterine environment.

Conclusions: Based on our findings, we hypothesise that differences in the level of four metabolites in the amniotic fluid may play role in the pathogenesis of DS. Defining their potential as biochemical pathogenic factors of DS requires further investigation of the biological pathways involving in the foetal development.

Key words: Down Syndrome; amniotic fluid; metabolomics

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INTRODUCTION

Chromosomal aberrations are the main cause of many congenital malformations. The most common is trisomy 21, also known as Down Syndrome (DS). It is estimated that 1:700 live births in the United States were affected with this chromosomal abnormality between 2010 and 2014 [1]. This is the most recent report on the incidence of DS, but according to the World Health Organization data, incidence rates have not changed to date. Individuals with DS are affected by many disorders, which may occur in different combinations. According to the National Institute of Child Health and Human Development, the most common problems include mental retardation, heart defects, vision problems, hearing loss, infections, hypothyroidism, blood disorders, hypotonia

(poor muscle tone), cervical spine abnormalities, disrupted sleep patterns and sleeping disorders, gum disease and dental problems, epilepsy, digestive problems, celiac disease and mental health and emotional problems [2]. Such a diversity of defects complicates the explanation of how an additional chromosome may affect the whole organism.

The newest “omics” technologies may serve as potentially useful tools in providing an explanation of molecular mechanisms leading from chromosomal aberration to observed malformations [3]. Metabolomics, in particular, which aims to measure all small molecules below 1000 Da, is a powerful tool, as it shows the current status of an organism. In the case of research on human beings, it is commonly used to compare individuals affected by a studied parameter (e.g.

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disease or treatment) with an appropriate control group [3]. Observed changes provide a comprehensive view of alterations in the metabolome caused by the studied factor. Consequently, metabolomics can be applied to study different aspects of disease and/or treatment including a search for diagnostic markers, explanation of disease pathogenesis, treatment strategy (personalized medicine) or monitoring of treatment efficacy. Nuclear magnetic resonance (NMR) or mass spectrometry (MS) are analytical techniques which are most commonly used in metabolomics research. To improve its sensitivity, MS is often used in combination with one of the separation techniques: liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). The methods complement one another as each provides information about different compound classes [4]. However, as a single analytical platform, LC-MS provides the highest metabolome coverage [5] and therefore it was used in our study.

Metabolomics has previously been used to evaluate mechanisms which are the basis of impaired development of a foetus with DS or to find new indicators which may improve the prenatal diagnostic process [3, 6–10]. Maternal blood has been analysed using LC-MS [3] and NMR [6]. A panel of fatty amides (palmitic amide, linoleamide and oleamide), which can be connected with foetal brain and central nervous system development, discriminated DS-affected maternal plasma from the plasma of healthy pregnant women [3]. In a similar study performed with the use of NMR, Bahado-Singh et al. [6] found that 2-hydroxybutyrate, 3-hydroxybutyrate, 2-hydroxyisovalerate, acetamide, acetone, carnitine, lactate, pyruvate and L-methylhistidine were increased while dimethylamine and methionine were decreased in the plasma of women with a DS-affected pregnancy. These compounds can also be connected with brain development (brain myelination), hypotonia, learning disability, seizure disorders, brain atrophy or oxidative stress. Maternal urine, biological material which can be obtained in an entirely non-invasive way, was also analysed using metabolomics [9, 10]. Using LC-MS, Trivedi et al. [9] found that dihydrouracil was significantly elevated in the urine of women with a DS-affected pregnancy. A similar study, but focused on DS, other chromosomal disorders and poor pregnancy outcomes was conducted by Diaz et al. using the NMR technique. In the study, urine levels of 2-ketoglutarate, 1-methylhistidine, 3-hydroxybutyrate, 4-OH-hippurate and dimethylamine were found to discriminate pregnant women carrying a foetus affected with a chromosomal disorder from the control group, but none of the significant metabolites were found to be specific to DS [10]. These studies provide some information regarding the way in which metabolic pathways are affected by the presence of DS in a foetus. To complete the picture, we focused on amniotic fluid (AF) in our study. AF is routinely used for karyotyping and was a biofluid

of choice for our research since it reflects the condition of both the mother and the foetus. AF composition changes dynamically during gestation. In early pregnancy, it contains a low amount of proteins and enzymes, which increases with foetal development (in mid- and late pregnancy) [7, 8]. AF was analysed using different “omics” platforms (metabolomics, proteomics and genomics) to investigate a variety of pregnancy-related abnormalities [8, 11–14]. Those in which a metabolomics approach was used have recently been reviewed by Bardanzellu et al. [15].

Objectives

The aim of the study was to explore metabolic pathways affected by the additional chromosome 21. A LC-MS-based metabolomic approach was utilised to study AF samples obtained from women with a DS-affected pregnancy and women carrying a foetus with normal karyotype.

MATERIAL AND METHODS

Study group

The study and control groups consisted of women who underwent routine amniocentesis between the 15th and 18th week of gestation at the Department of Reproduction and Gynecological Endocrinology of the Medical University of Bialystok, Poland. Exclusion criteria were as follows: chronic or acute diseases, hormonal treatment, anti-inflammatory treatment, high-risk pregnancy or preterm delivery in the patient’s medical history. All participants were informed about potential risks prior to the procedure and received relevant information regarding the study. Moreover, study participants were matched according to the course of pregnancy and BMI values. Details of the study group are presented in Table 1. Following karyotype test results analysis, 13 women carrying fetuses affected with DS and 13 women with healthy fetuses were enrolled in the study. The study was approved by the Ethics Committee of the Medical University of Bialystok (No. R-I-002/134/2018). Prior to inclusion in the study, all participants signed informed consent forms.

Materials

Deionised water was obtained in-house using the Milli-Q Integral 3 system (Millipore SAS, Molsheim, France). LC-MS grade acetonitrile (ACN), methanol (MeOH) and formic acid (FA) were purchased from Sigma-Aldrich Chemie GmbH

Table 1. The characteristics of the study participants

	case (n = 13)	control (n = 13)
Maternal age (mean ± SD)	38 ± 4 years	36 ± 5 years
Maternal BMI (mean ± SD)	24 ± 3.8	24 ± 1.8
Gestation age (mean ± SD)	16 ± 1 weeks	16 ± 1 weeks

SD — standard deviation; BMI — body mass index

(Steinheim, Germany). LC grade ethanol was purchased from POCH (Gliwice, Poland). The API-TOF reference mass solution kit (G1969-850001) and tuning solutions, ESI-L low concentration tuning mix (G1969-850000) and ESI-TOF Biopolymer Analysis reference masses (G1969-850003) were purchased from Agilent Technologies (Santa Clara, California, USA).

Sample preparation

The obtained 10 mL of AF was divided into smaller portions and stored at -80°C until the day of analysis. On the day of analysis, samples were thawed on ice and vortex-mixed. Then, to extract metabolites and remove proteins, 100 μL of methanol and ethanol mixture (1:1) was added to 100 μL of the sample. It was again vortex-mixed (1 min) and incubated on ice for 10 min. After that time, samples were centrifuged at $21000 \times g$ for 20 min at 4°C . The obtained pellet was removed and the supernatant was filtered through a 0.22 μm nylon filter into glass vials. Quality control (QC) samples were prepared by mixing equal volumes of all analysed samples. The mixture was extracted following the same procedure as that used with other samples and analysed together in the intervals.

Metabolic fingerprinting

Samples were analysed using the 1290 Infinity UHPLC system combined with the 6550 QTOF mass spectrometer (both Agilent Technologies). Chromatographic separation was obtained with a Poroshell 120 EC-C18 column (3.0×100 mm, 2.7 μm particle size). Deionised water (phase A) and acetonitrile (phase B), both with 0.1 % formic acid, were used as mobile phases. Proportion of mobile phases was changing with the following gradient: analyses commenced with 1% of phase B and it was kept for 1 min, then the gradient started reaching 100% of B at 10 min. After that, the gradient moved back to the starting condition in 10.1 min and the system was re-equilibrated before the next injection for the next 5 min. Electrospray ionisation (ESI) was used and the samples were analysed separately in both positive and negative ion modes. The mass spectrometer was operated in full scan mode in the range 50–1000 m/z at a scan rate of 2 spectra per second. To ensure high accuracy of m/z measurements, reference mass solution was continuously delivered by an isocratic pump. The following compounds were observed: protonated purine (m/z 121.0509), protonated hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine (m/z 922.0098) in positive ion mode; proton abstracted purine (m/z 119.036) and formate adduct of HP-921 (m/z 966.0007) in negative ion mode.

Data processing and statistical analysis

Raw data collected by analytical instrumentation were cleaned of background noise and unrelated ions by the molecular feature extraction (MFE) tool in Mass Hunter

Qualitative Analysis Software (B.07.00, Agilent, Santa Clara, CA, USA). The MFE creates a list of all possible components described by mass, retention time (RT) and abundance. To identify co-eluting adducts of the same feature, the following adduct settings were applied: +H, +Na, +K for positive ion mode, and –H, +HCOO, +Cl for negative ion mode. Dehydration neutral losses were also allowed in both ionisation modes. Obtained data were aligned allowing for 1% shift in RT and 15 ppm variation in mass measurement. To maintain proper data quality, a quality assurance (QA) protocol was applied. Only features detected in more than 50% of QC samples and with adequate repeatability ($\text{CV} < 25\%$) were kept. Additionally, data were filtered based on their presence in studied groups, i.e. metabolites present in at least 80% of the samples from at least one of the groups were kept. Sample alignment, data filtering and QA protocol were performed using Mass Profiler Professional 12.6.1 (Agilent, Santa Clara, CA, USA). To check data quality based on the projection of QC samples, principal component analysis (PCA) was used. To visualize differences in AF metabolic profiles between the studied groups, partial least squares discriminant analysis (PLS-DA) was used. SIMCA P+ 13.0.3 (Umetrics) was used to create multivariate plots. To select metabolites discriminating studied groups, univariate statistical analysis was used. Depending on normality of data distribution (checked using the Shapiro-Wilk test), the Student t test (normally distributed data) or the Mann-Whitney U test (data without a normal distribution) were applied. Obtained p -values were corrected by the Benjamini-Hochberg false discovery rate (FDR). Values lower than 0.05 were considered significant.

Statistically significant compounds were putatively identified by searching for potential hits in online databases (METLIN, HMDB and LIPIDMAPS), accessed through CEU Mass Mediator (<http://ceumass.eps.uspceu.es/>) [16]. The identity of metabolites was confirmed by matching the experimental MS/MS spectra to MS/MS spectra from databases. Experiments were repeated with chromatographic conditions identical to those in the primary analysis. Ions were targeted for collision-induced dissociation (CID) fragmentation on the fly based on previously determined accurate mass and RT.

RESULTS

As a result of the QA procedure and data filtering, 1410 and 940 metabolic features remained in positive and negative ion modes, respectively. Based on these data, PCA models were created showing a close clustering of QC samples (Fig. 1), which indicates good data quality. The same data were used to build PLS-DA models. As shown in Figure 2, study groups can be discriminated on the basis of the metabolic profiles obtained in positive (panel A) and negative (panel B) ion modes. Statistical analysis and metabolite identification were performed as described in the

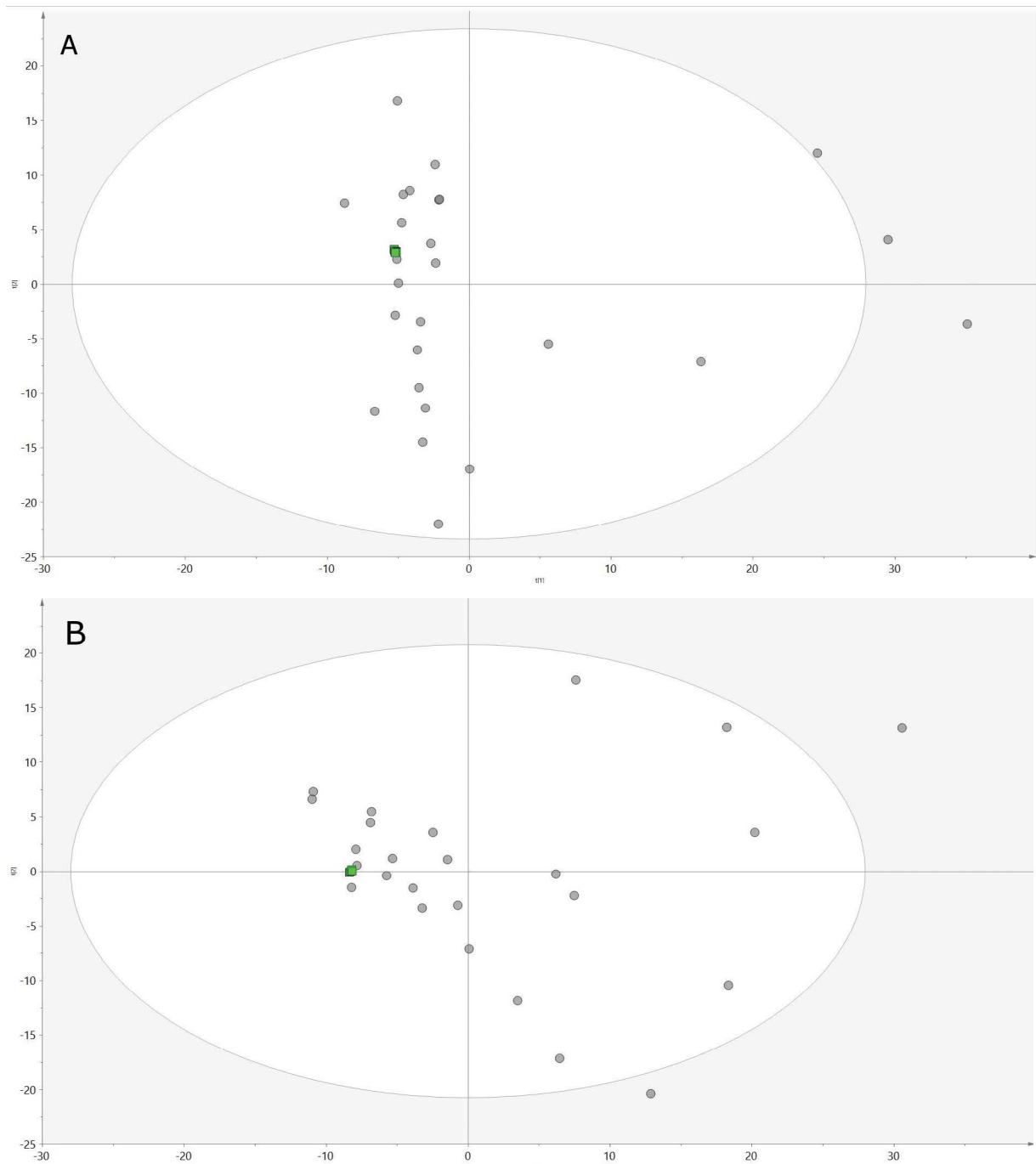


Figure 1. Classification of quality control samples (marked in green) on a principal component analysis (PCA) model. Panel A shows a PCA model based on data generated in positive while panel B in negative ion mode. Data was log transformed and Ctr scaled

Materials and Methods section. Identified metabolites are presented in Table 2.

DISCUSSION

To date, alterations in metabolic pathways evoked by foetal DS have been studied with the metabolomic approach using, primarily, maternal blood or urine samples [3, 6, 9, 10, 17]. Therefore, data obtained from our analysis of AF samples may

complement previous reports on the subject. AF has been analysed by Liu et. al. [7] and Huang et. al. [8] who observed similar changes in the levels of cortisol, free amino acids (arginine, histidine and glutamate) and pregnenolone sulfate. Liu et al. [7] used different types of chromatographic separation, which resulted in a discovery of a larger number of differentiating compounds (151 metabolites). These metabolites were grouped into the following metabolic pathways: *gamma*-glu-

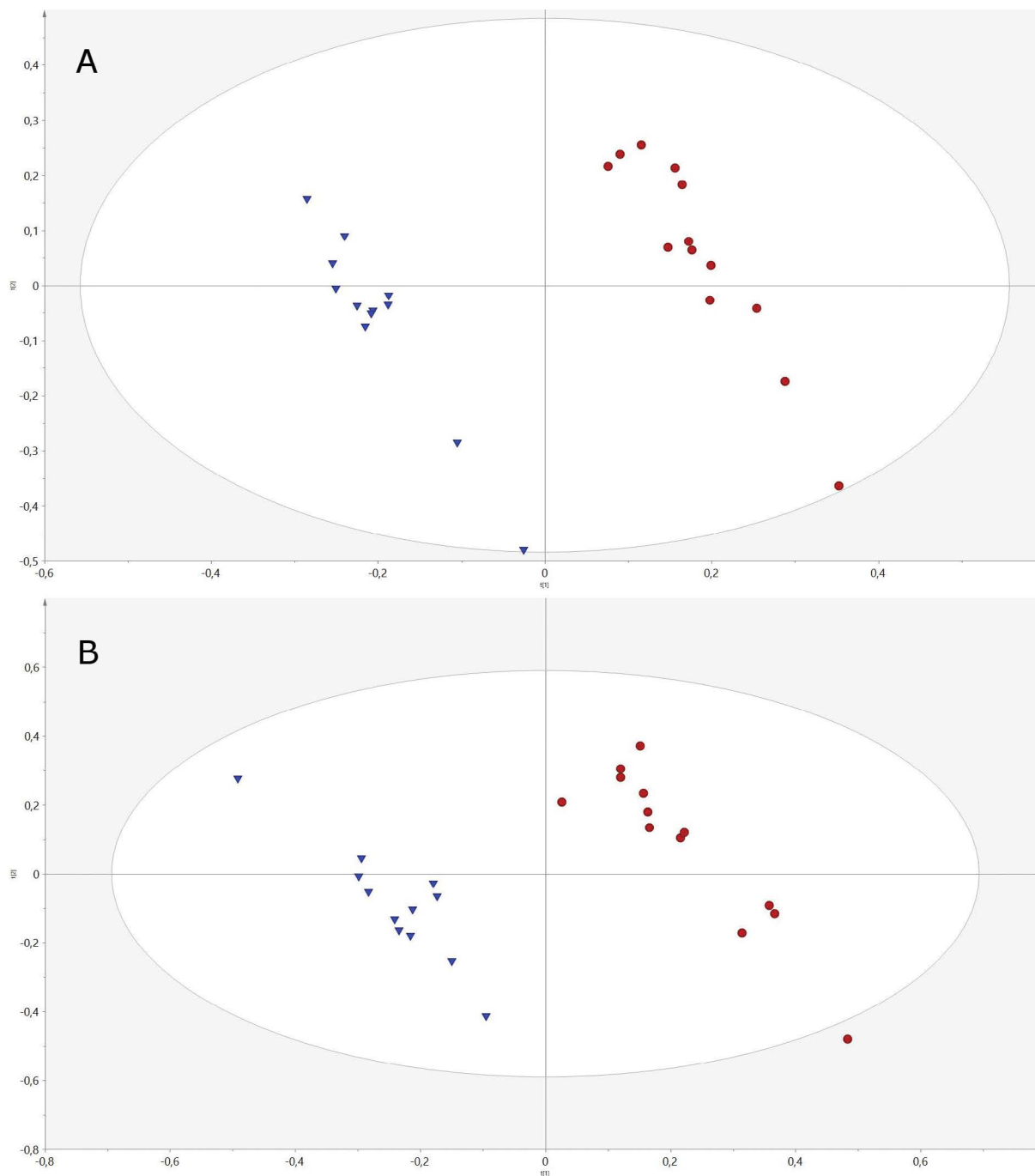


Figure 2. Partial least squares discriminant analysis (PLS-DA) score plots showing separation between patients with foetal Down syndrome and control group. PLS-DA models based on data generated in positive (Panel A, $R^2 = 0.991$, $Q^2 = 0.509$, Par scaling and log-transformed data) and in negative (Panel B, $R^2 = 0.972$, $Q^2 = 0.58$, Par scaling, log transformed) ion modes; ● DS pregnancy; ▼ healthy pregnancy

Table 2. Significant metabolites

Compound name	Monoisotopic mass	Ionisation mode	Retention time	p value	Change
Methylhistidine	169.0855	positive	0.97	0.0484	-27.09
Hexanoylcarnitine	259.1787	positive	4.65	0.0464	-38.66
Diacetylspermine	286.2369	positive	1.09	0.0464	+42.92
p-cresol sulfate	188.0146	negative	4.73	0.021	-40.94

tamyl amino acids metabolism, phospholipid metabolism, fatty acid and dicarboxylate, pentose metabolism, glycogen metabolism, disaccharides and oligosaccharides, fructose, mannose and galactose metabolism, aminosugar metabolism and tricarboxylic acid cycle. In the study by Huang et al., a smaller number of metabolites differed significantly, but the obtained results were confirmed with a validation set. Moreover, some of the metabolites, *i.e.* coproporphyrin-III, taurochenodeoxycholate, taurocholate and glycocholic acid, were not reported by Liu et al. [7].

In our study, similarly to the study by Liu et al. [7], an increase in diacetylspermine was observed. This metabolite has been indicated as a factor connected with maternal exposure to nicotine [18] and as a marker of breast and colorectal cancer [19], but its association with metabolic changes evoked by foetal DS has not been elucidated [7].

According to our knowledge, the present study is the first to demonstrate differences in AF composition between DS-affected pregnancies and pregnancies with fetuses with normal karyotype in the Caucasian race. Both studies mentioned above [7, 8] concern Asian people, but as shown by Taylor et al. [20], race of the study group is an important factor affecting the results. Observed interracial differences may not only have a genetic background, but also could be the result of specific maternal and dietary habits as well as environmental factors. A Western-style diet, based on processed food, impacts both the foetus (amniotic fluid volume and composition [21]) and the mother (gut microbiota composition [22]). A high-fat diet promotes excessive weight gain or can even induce maternal obesity, which may result in hypertension or preterm delivery [21]. Neither Liu et al. [7] nor Huang et al. [8] included information on the BMI of study participants. The hypothesis that research results may be related to the ethnicity of the population studied can be supported by our observation of changes in *p*-cresol sulfate. While *p*-cresol is a product of bacterial fermentation of aromatic amino acids [23], *p*-cresol sulfate is produced in colonic mucosa and the liver as a result of detoxification of *p*-cresol. The dogma of sterile intrauterine environment has recently been challenged [24–28]. It has postulated that bacterial colonisation of the foetus, amniotic fluid and placenta in utero potentially comes from the maternal oral cavity via the bloodstream, from vagina via translocation across the chorionic plate or from the maternal intestine [25]. The last source is particularly interesting when we try to explain differences observed in *p*-cresol sulfate. As mentioned above, a high-fat diet (which is a typical Western-style diet) may affect maternal gut microbiota and may be associated with poor maternal health outcomes. Our observation of microbial metabolite presence in AF may provide additional confirmation that the intrauterine environment is not sterile. Moreover, the presence of bacteria in utero allows for the prenatal development of foetal gut microbiota [27–31]. Borre

et al. [26] highlighted parallels between microbiota and brain (or more generally, the nervous system) development. Their theory is related to the brain-gut axis which coordinates interactions between gut microbiota and the central nervous system in postnatal life (CNS).

Our study was the first to detect methylhistidine in AF. It is excreted with urine, which can explain its presence in AF. Methylhistidine was found to be decreased in the AF of DS-affected pregnancies. It is a product of methylation of histidine, which occurs mainly in skeletal muscle cells during methylation of actin and myosin. Therefore, the observed presence of methylhistidine can be connected with hypotonia, a neuromuscular disorder that affects almost all individuals with DS [32]. In the third trimester, the amount of methylhistidine in maternal urine increases, which is connected with the protein cost of pregnancy [33]. Moreover, it has been demonstrated that methylhistidine concentration is elevated in the urine of women carrying fetuses affected by a chromosomal disorder. The increase was observed in the second trimester before the expected rise in a methylhistidine level related to the natural course of pregnancy [10]. Interestingly, Bahado-Singh et al. also observed elevated methylhistidine levels in the serum of women with a DS-affected pregnancy in the first trimester (11th–13th week of gestation) [6]. Similar changes in methylhistidine concentration were observed in maternal blood when women with a pregnancy affected with trisomy 18 were studied [34].

Another metabolite whose concentration in AF was decreased in our study group in comparison to the control group was hexanoylcarnitine. Long-chain fatty acids in the form of acylcarnitines are transported to mitochondria for β -oxidation. However, apart from this function, this group of metabolites has a neuroprotective role and affects brain metabolism [35]. Therefore, the observed changes in hexanoylcarnitine concentration may reflect on foetal brain and CNS development. Parfieniuk et al. and Bahado-Singh et al. have also observed significant differences in the level of particular acylcarnitines when blood metabolic profiles of women carrying a foetus affected with DS were compared with those of women carrying a foetus with normal karyotype [3, 6]. Despite the fact that carnitines are excreted with urine, no statistically significant differences in their levels were found when similar studies on urine samples from DS-affected and healthy pregnancies were performed [9, 10].

CONCLUSIONS

The present study increases current knowledge regarding the impact of an extra copy of chromosome 21 on metabolic imbalance. The compounds detected in our study may be related to disturbed foetal brain and CNS development. During the second trimester, the composition of AF changes and the concentration of many molecules increases as the foetus

excretes them. A decrease in the majority of significant metabolites observed in our study may be the result of differences in the pathological pathways of disturbed foetal development.

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Conflict of interest

None.

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