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# Study on the Mechanism of Early Delivery by Multi-Omics Analysis of Metabolites, Elements and Bacterial Flora in Amniotic Fluid

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## ■ Abstract

A gas chromatograph mass spectrometer (GCMS-TQ™8040 NX) and an energy-dispersive X-ray fluorescence spectrometer (EDX-7200) were used to analyze metabolites (488 components) and elements (78 elements) contained in the amniotic fluid of pregnant women. These data were combined with amniotic fluid microbiota (approximately 250 species of microorganisms) measured using a miniature next-generation sequencer (MinION Mk1C System, Oxford Nanopore Technologies, Inc.) and 16 items of labor data (e.g., neonatal weight) to analyze differences between preterm and normal births. Volcano plot and metabolic pathway analyses were performed using Multi-omics Analysis Package based on the 740 items detected. In this application, we introduce an example of multi-omics analysis conducted by gender and time series.


## 1. Introduction

Preterm delivery refers to delivery at a gestational age of 22 weeks or less than 37 weeks, which can have a significant impact on the health and development of the newborn and the life of the family. This problem occurs worldwide: in 2020, 1/10 of all births were preterm[1)] (Fig. 1). Prematurity is the leading cause of early childhood mortality, so there is an urgent need to promote research on preterm birth.

However, the global rate of premature birth reduction from 2010 to 2020 was only 0.14 %, indicating that the birth cycle has not improved. It is known that preterm birth is closely related to amniotic fluid, and it is used as a research object in preterm birth[2)]. Amniotic fluid is approximately 500 mL of water, contains fetal urine, sloughed cells, inflammatory substances, etc., and is a valuable source of information for understanding fetal growth and health[3)]. Measuring metabolites in amniotic fluid helps assess the fetus's metabolic activity and nutritional status. Elemental measurements can also determine the extent of fetal development and environmental exposure.

It is also essential to measure the bacterial flora in the amniotic fluid. It has been found that about 80 % of preterm births occur in pregnant women who have a chain of bacterial infections from the vagina to the uterus and from the uterus to the amniotic fluid [4)].

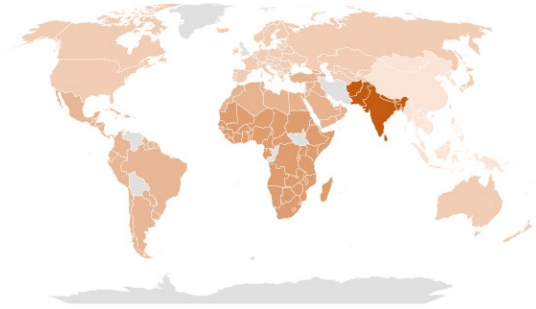

Fig. 1 Preterm birth rate by country/region in 2020
(the darker the red, the higher the preterm birth rate)
*13.2 % in high areas and 7.9 % in low areas

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In this study, we used a gas chromatograph mass spectrometer GCMS-TQ8040 NX, an energy-dispersive X-ray fluorescence spectrometer EDX-7200, and a miniature next-generation sequencer MinION Mk1C to measure primary metabolites, elements, and flora in the amniotic fluid of preterm and term pregnant women (Fig. 2). These data were combined with 16 birth delivery data items (e.g., female age, newborn weight, etc.), and the differences in amniotic fluid content between preterm and term births were visualized using Multi-omics Analysis Package.

## 2. Experimental

The samples were amniotic fluid from preterm (n=3) and term (n=3) women. A total of 796 compounds, elements, and bacterial species were measured using the following 3 instruments to evaluate amniotic fluid from multiple angles (multi-omics analysis) (Fig. 3).

### 1) Gas chromatograph mass spectrometer

488 primary metabolites, including organic acids, sugars, nucleic acids, fatty acids, and amino acids, were measured using GCMS-TQ8040 NX and Smart Metabolites Database™ Ver. 2. Approximately 400 primary metabolites were detected in each sample. The area value of the metabolite detected in each sample was subtracted from the area value detected in the blank water sample and then corrected with the internal standard 2-isopropylmalic acid.

Fig. 2 EDX-7200 (left), MinION (top), GCMS-TQ™8040 NX (right)

### 2) Energy dispersive X-ray fluorescence Analyzer

300 µL of each inactivated sample was dispensed into a sample container and measured under a He gas atmosphere. The analysis targets 78 elements (excluding rare gases), ranging in atomic number from 11th Na (sodium) to 92nd U (uranium). The lower limit of detection varies depending on the element. Still, it ranges from a few ppm to a few hundred ppm, and we analyzed the elements in amniotic fluid. Six elements (Cl, K, Ca, S, P, Br) were detected in each sample.

### 3) Next-generation sequencer

The samples were lysed with the QIAamp DNA Microbiome Kit (QIAGEN), followed by DNA extraction, and the entire length of the 16 S rRNA gene was cloned into the universal primer 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). The library was prepared according to Nanopore's protocol, and Amplicon DNA was sequenced using MinION Mk1C. 396 bacterial species were measured, approximately 20 were identified in each subject, and the relative quantification value was calculated.

Approximately 700 explanatory variables (compounds, elements, and species) found were loaded into Multi-omics Analysis Package for statistical analysis. The multi-omics analysis package provides software for principal component analysis, rank cluster analysis, box plot analysis, volcano plot analysis, and metabolic pathway analysis (correlation analysis and time series analysis on the metabolic pathway map) (Fig. 4).

Fig. 4 Multi-omics Analysis Package (Garuda)

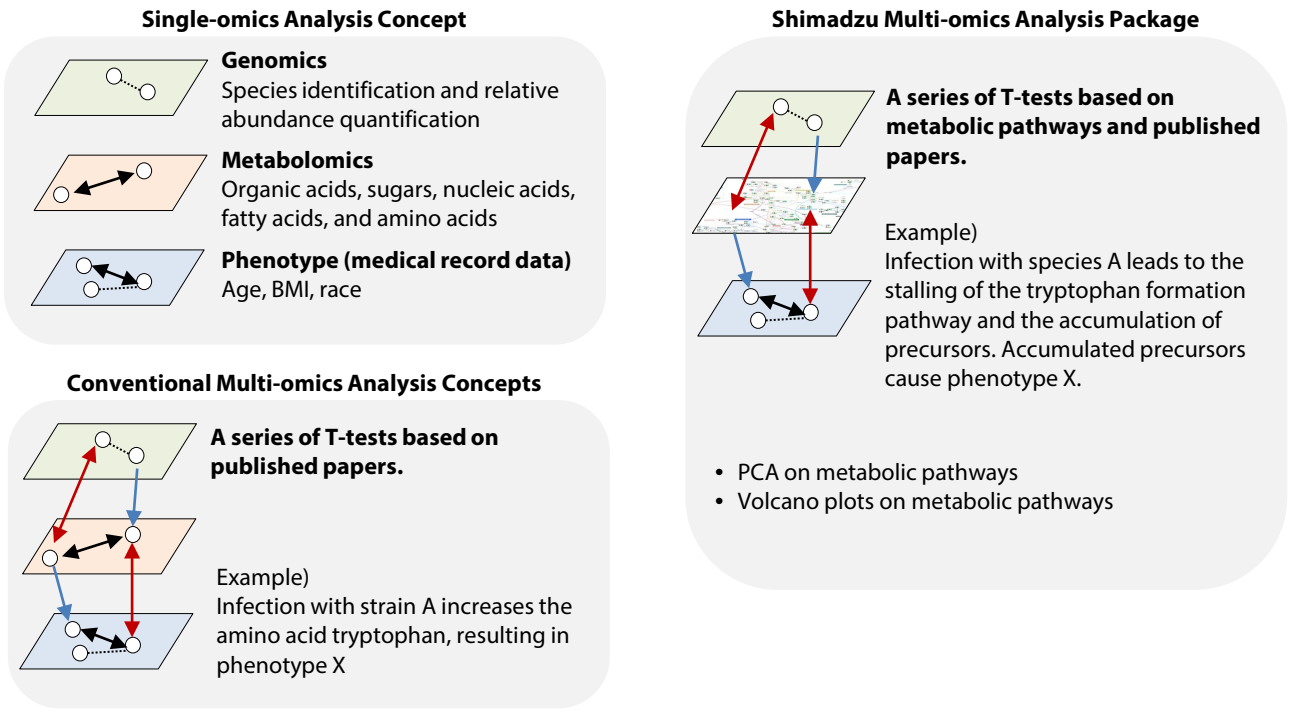


Fig. 3 Concepts for single-omics and multi-omics analyses

## 3. Results

Approximately 700 explanatory variables detected in the amniotic fluid of preterm pregnant women (n=3) and term pregnant women (n=3) were analyzed by Volcano plot (Fig. 5). The results showed that there were few variables that were specifically high in the amniotic fluid of term pregnant women, and there were many test items that were specifically high in the amniotic fluid of preterm pregnant women. Amniotic fluid from preterm pregnant women was found to contain specifically high concentrations of lipids and fatty acids, including oleic acid (p value 0.001), margaric acid (p value 0.04), batyl alcohol (p value 0.04), mystiphosphoric acid (p value 0.005), and lauric acid (p value 0.03).

In addition, the number of copies of the 16 S ribosomal RNA gene (copies/μL) was found to be particularly high in the amniotic fluid of preterm pregnant women, suggesting a possible relationship between the number of bacteria and the delivery cycle. Therefore, the results of the next-generation sequencer were narrowed down to the major bacteria (Fig. 6).

The results showed that ureaplasma bacteria were highly detected in the amniotic fluid of subjects A and B who gave birth prematurely. However, subject C, who also gave birth prematurely, was not infected with the bacterium, and the six subjects were classified into three groups: ureaplasma-infected, germ-free, and mixed-infection. Subjects D, who had mixed infections but delivered at term, had higher levels of phosphorus than the other subjects (Fig. 5). Phosphorus (P) in amniotic fluid plays a role in determining the antibacterial effect of trace amounts of zinc, but in subject D, zinc was not detected, and the antibacterial effect is predicted to be low5). A weak positive correlation was also found between the concentration of bromine (Br) and the number of ureaplasma bacteria.

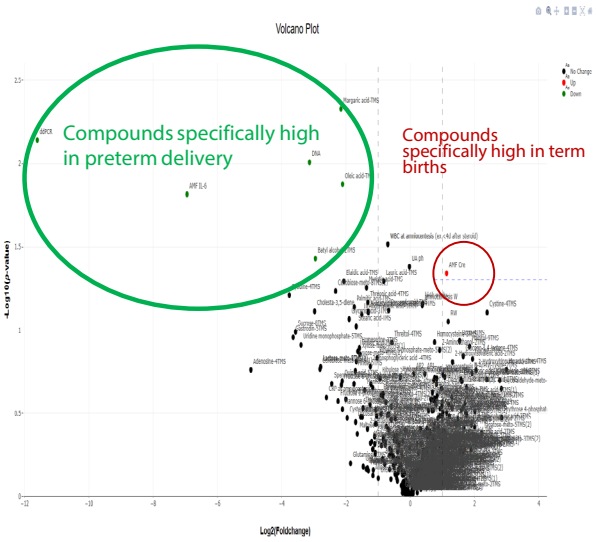


Fig. 5 Volcano plot analysis of preterm (n=3) and term (n=3) births
(About 700 variables for metabolites, elements, species, and delivery data)

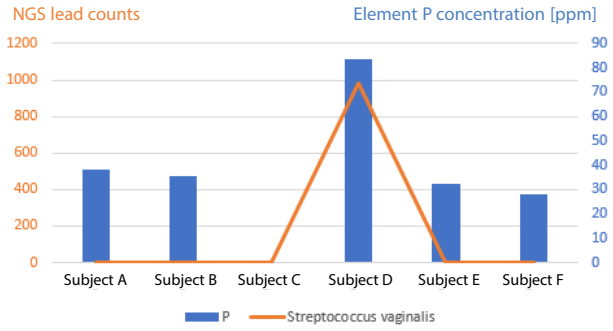


Fig. 7 Amniotic fluid phosphorus levels (Blue) and lead counts of Streptococcus basinalis (Orange)

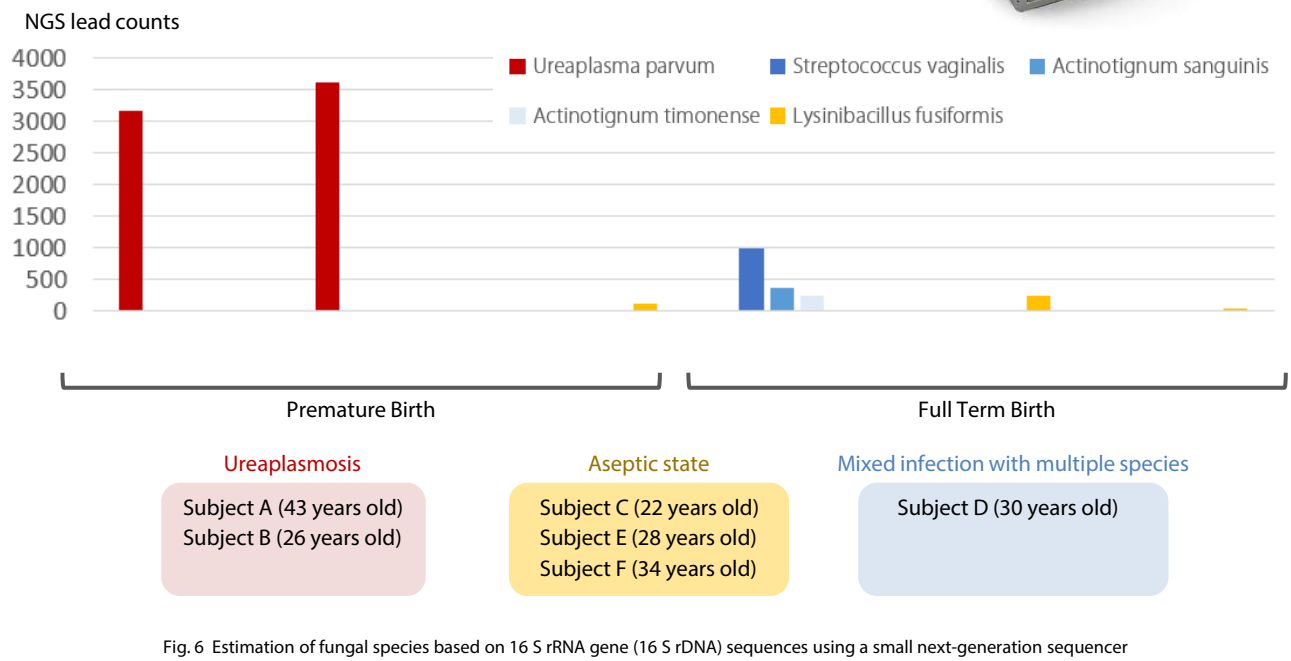


Fig. 6 Estimation of fungal species based on 16 S rRNA gene (16 S rDNA) sequences using a small next-generation sequencer
(Sample: Amniotic fluid, only selected major bacteria)

Based on the results of the next-generation sequencer, six subjects were classified into three groups: ureaplasma-infected, germ-free, and mixed-infection, and metabolic pathway analysis was performed. To perform a comprehensive correlation analysis, in addition to metabolic pathways, we added delivery data such as elements detected by EDXRF, major species detected by next-generation sequencers, and newborn weight to a single metabolic pathway map (Fig. 8). In the bar graph, red indicates ureaplasma-infected persons, yellow indicates uninfected persons, and blue indicates mixed infections. The color of each item box indicates that the items that are more common only in ureaplasma-infected persons are red, and conversely, the items that are less common only in ureaplasma-infected persons are blue, and pastels indicate no correlation.

It was found that the glycolytic metabolic pathway (energy source generation pathway) was activated in individuals infected with ureaplasma bacteria, and products were detected higher than in other subjects. Assuming that this metabolic pathway is derived from fetal cells, it means that there is an increase in the number of substrates that produce ATP, a high-energy compound that occurs in cells. The accumulation of intermediate metabolites in the pyrimidine base metabolism pathway was also observed. Since pyrimidine bases are catabolized into sugar and lipid metabolic pathways, an increase in intermediates indicates an increase in products, suggesting that the metabolic pathway is activated.

In addition to metabolite accumulation, the multi-omics analysis package graphically lists the ratio of product to substrate and the reciprocal ratio of substrate to product to perform correlation analysis (Fig. 9 on the next page). By projecting these ratios as new variables into the rank cluster analysis, it becomes a tool to confirm the clustering process and to elucidate unknown metabolic pathways (Fig. 10 on the next page).

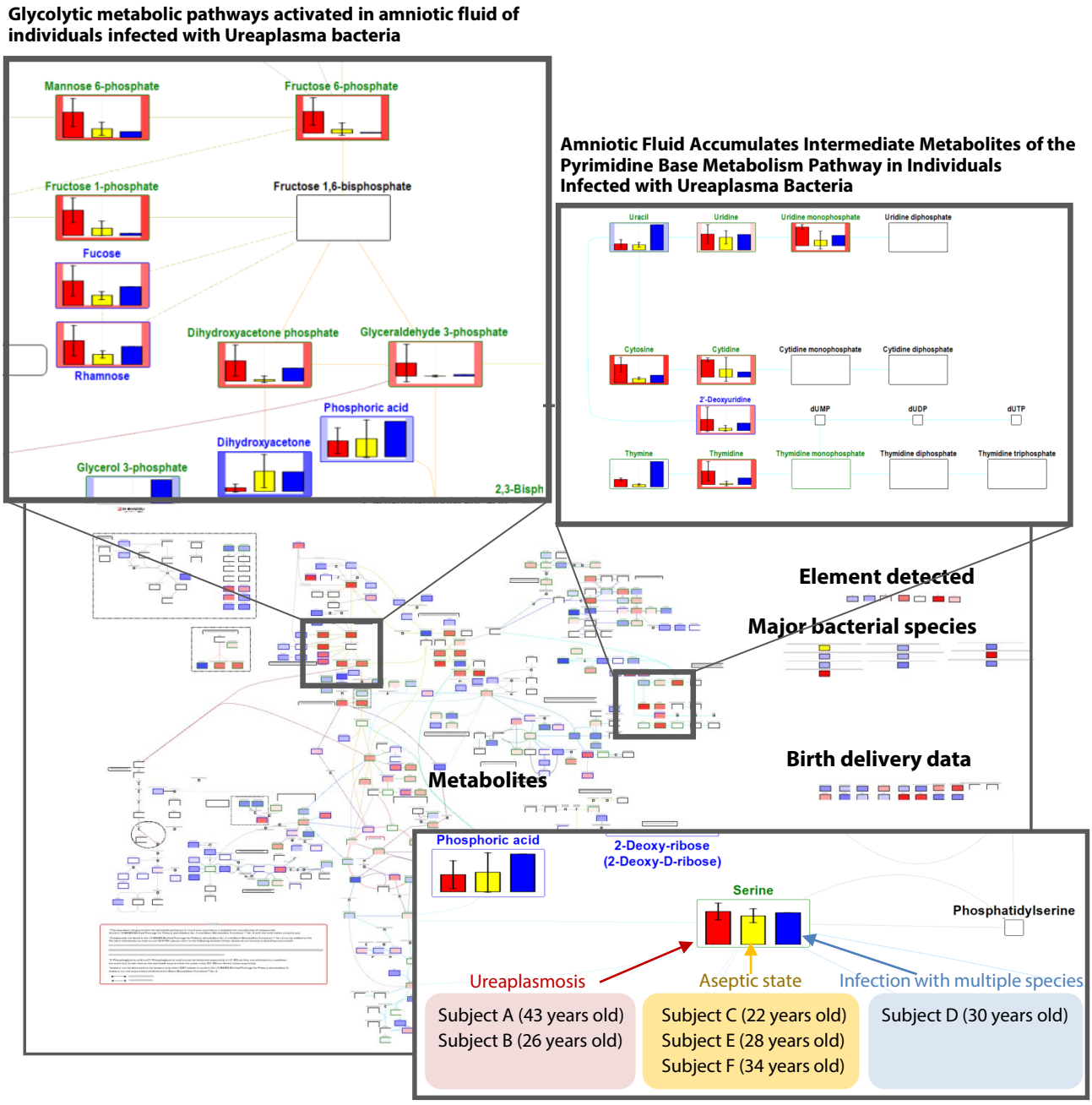


Fig. 8 Metabolic pathway analysis of six subjects divided into three groups by NGS
Bars in red are ureaplasma-infected individuals, yellow are uninfected individuals, and blue are mixed infections.
The color of each metabolite box is red if it is high for those with ureaplasma infection only, blue if it is low for those with ureaplasma infection only, and pastels if there is no correlation.

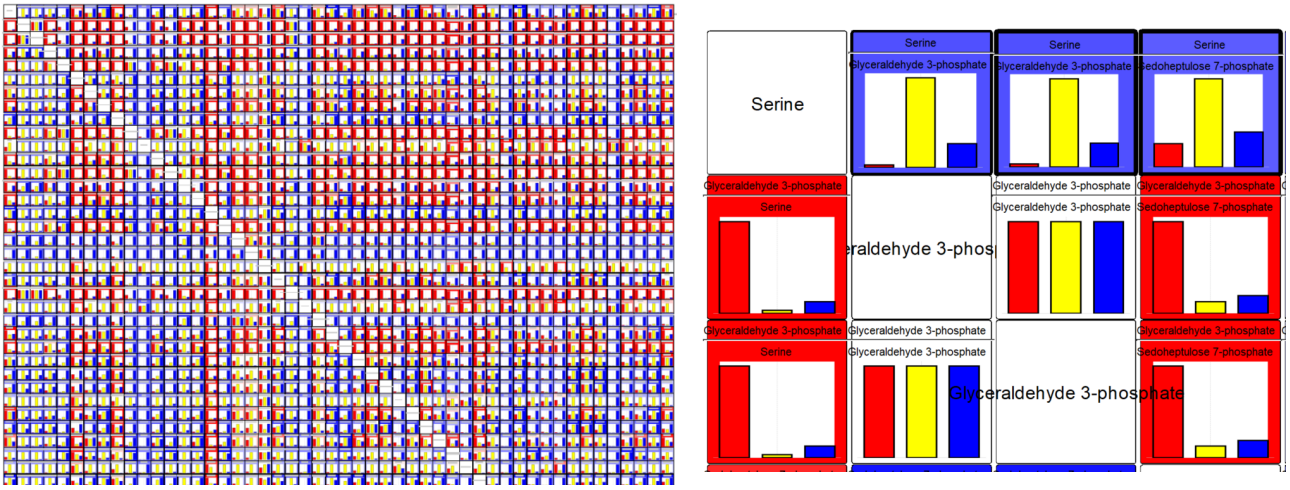


Fig. 9 Metabolic pathway analysis of six subjects divided into three groups by NGS
(visualizing correlations using the ratio of product/substrate and its inverse, substrate/product, as variables)
Three bars show ureaplasma-infected individuals on the left, uninfected individuals in the middle, and mixed infections on the right.
The color of each box is red if it is high for those with ureaplasma infection, blue if it is low for those with ureaplasma infection, and pastels if no correlation.
*Metabolites with similar chemical structures, such as substrates and products, are assumed to have similar ionization efficiencies.

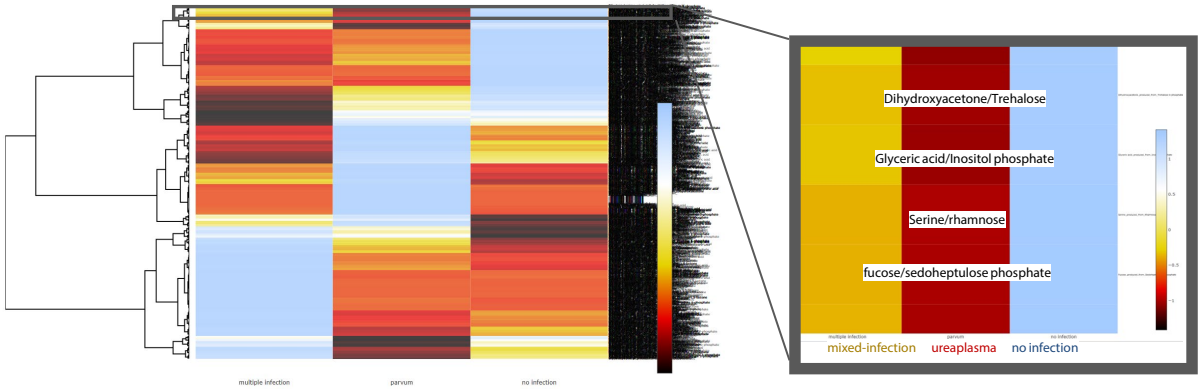


Fig. 10 Class cluster analysis using product/substrate and its reciprocal substrate/product as variables to search for clusters of metabolite pairs with similar behavior

## 4. Conclusion

Amniotic fluid from 6 pregnant women (3 preterm births and 3 term births) was measured using a next-generation sequencer (MinION Systems, Oxford Nanopore Technologies, Inc.), a gas chromatography-mass spectrometer (GCMS-TQ8040 NX), and an energy-dispersive X-ray fluorescence spectrometer (EDX-7200) to evaluate the intestinal microbiota (approximately 250 species of microorganisms), metabolites (approximately 500 components), and elements (approximately 80 elements). Metabolic pathway analysis was performed using Multi-omics Analysis Package based on approximately 700 items detected.

In metabolic pathway analysis, not only the accumulated amount of metabolites, but also the ratio of product/substrate or its reciprocal substrate/product was automatically created in Multi-omics Analysis Package and used as a new variable to be read into class cluster analysis, etc., thereby enabling statistical analysis from a perspective different from the accumulated amount.

<References>

1) Born too soon: decade of action on preterm birth, WHO, accessed on April 9th, 2024

2) 羊水の問題 [amniotic fluid problem], MSD, accessed on April 9th, 2024

3) 羊水とは？赤ちゃんを守る重要な役割 [What is amniotic fluid? important role in protecting babies], Moony, accessed on April 9th, 2024

4) 早産になる原因となりやすい人の特長 [Characteristics of people who are prone to premature birth], NIPT Japan, accessed on April 9th, 2024

5) Phosphate-to-zinc ratio as a predictor of bacterial growth-inhibitory activity, PubMed, accessed on April 9th, 2024



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