Metabolomic Analysis of the Cerebrospinal Fluid in Latent Syphilis and Neurosyphilis Patients

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Abstract

Purpose: The infection rate of syphilis continues to rise, and the difficulty in detecting and treating neurosyphilis promptly needs to be resolved. The metabolic profiles of cerebrospinal fluid (CSF) of different patients were analyzed to understand the pathogenesis of syphilis better.

Method: The metabolic profiles of 88 CSF samples from patients were analyzed by UPLC-Q Exactive-MS. The experimental process was evaluated by PCA, PLS-DA, and HCA. T-test statistics were used to compare levels of metabolites to determine significant differences between groups. Pathway analysis was based on the KEGG database.

Result: In total, 272 metabolites based on 3937 features obtained in ESI- mode and 252 metabolites based on 3799 features in ESI+ mode were identified. A clear separation between latent syphilis and neurosyphilis was found. Levels of lipid and linoleic acid metabolites, such as 9-OxoODE and 9,10,13-TriHOME, were increased in syphilis patients. In patients with neurosyphilis, significant changes in levels of 5-hydroxy-L-tryptophan (5-HTP) and acetyl-N-formyl-5-methoxykynurenamine (AFMK) in the tryptophan-kynurenine pathway were also detected. Only one metabolite, theophylline, differed significantly between symptomatic and asymptomatic neurosyphilis patients. Additionally, KEGG analysis revealed significant enrichment of tryptophan metabolism pathways, indicating a high correlation between tryptophan metabolism and syphilis symptoms.

Conclusions: Levels of linoleic acid metabolites, 5-HTP, AFMK and theophylline were significantly altered in different patients. The role of these differential metabolites in the development of syphilis is worthy of further exploration, probably improving the treatment and diagnosis of neurosyphilis and occult syphilis in the future.

1. Introduction

Syphilis is a sexually transmitted disease (STD) caused by *Treponema pallidum* associated with significant complications if left untreated and can facilitate the transmission and acquisition of HIV infection. Without effective treatment, *T. pallidum* usually invades the central nervous system and causes neurosyphilis [1]. Neurosyphilis used to be a complicated disease in clinical research. With the discovery of penicillin, the prevalence of neurosyphilis and syphilis was controlled [2]. However, since 2000, the number of cases of syphilis has begun to increase again, with more than 100,000 new cases in 2018 alone; by contrast, only approximately 60,000 new cases of gonorrhoea occurred in 2018 [3]. The number of patients with neurosyphilis has also risen with the resurgence of syphilis. Merritt et al. reported that approximately 30% of all patients developed neurosyphilis, of whom 30% were asymptomatic [4].

Latent syphilis and neurosyphilis are difficult to distinguish when the early clinical symptoms are not obvious, which is the best treatment time. Nevertheless, diagnosis is complicated, and there is no gold standard. A neurosyphilis diagnosis is mainly based on clinical manifestations, specific and nonspecific serological tests for syphilis, abnormal cerebrospinal fluid (CSF), and occasionally neuroimaging [1,5].

Serology of neurosyphilis is usually detected by traditional methods, such as the nontreponemal test (NTT) using the Venereal Disease Research Laboratory (VDRL) or rapid plasma reagin (RPR), and treponemal tests, such as the *T. pallidum* agglutination test (TPPA), to confirm positive results [2,6]. Even this negative result does not entirely rule out neurosyphilis, as VDRL may be negative in 30% to 70% of cases [7]. Therefore, more specific and sensitive diagnostic markers should be found.

Metabolomics, which can directly reflect altered metabolites and their interaction with various stimulating factors, has great potential in disease screening and diagnosis biomarker identification. CSF is a biological fluid that is greatly affected by the dysfunction of the central nervous system, and analysis of CSF can well reflect changes in the nervous and biochemical states of the body [8,9]. Indeed, CSF metabolomics has been widely used in various neurodegenerative diseases and brain tumours, and studies have reported potential CSF diagnostic markers for Alzheimer's disease and malignant glioma [10,11]. Nevertheless, the use of CSF-based metabolites for a probable diagnosis of neurosyphilis remains mostly unexplored.

In this study, UPLC-Q Exactive-MS was conducted to analyze the metabolic profiles of CSF from 88 patients with syphilis, neurosyphilis, and non-syphilis, and more than 400 metabolites were identified in two modes. By evaluating the original data by multistatistical analysis, significant differences in the levels of CSF metabolites between syphilis and neurosyphilis were detected. T-test analysis showed that 20 metabolites differed between syphilis and non-syphilis patients, with five between neurosyphilis patients and latent syphilis patients. Only one metabolite, theophylline, was significantly different between symptomatic and asymptomatic neurosyphilis patients. The result may reveal the potential molecular mechanisms associated with syphilis and neurosyphilis, which may promote the development of syphilis biomarkers.

2. Materials And Methods

2.1 Participants

Informed consent was obtained from all subjects in this study. All experiments were performed following the approved guidelines. The patients included those with latent syphilis (Y, n=39), those with asymptomatic neurosyphilis (W, n=9), and those with symptomatic neurosyphilis (Z, n=33) as well as non-syphilis patients (F, n=7). Briefly, neurosyphilis was defined based on positive treponemal test results and the toluidine red unheated serum test (TRUSRT) [12,13]. Patients were considered to have symptomatic neurosyphilis if they have obvious clinical symptoms of neurosyphilis, such as meningitis, stroke, acute changes in mental status, abnormal hearing or vision; neurosyphilis patients with no neurological symptoms have asymptomatic neurosyphilis. Latent syphilis was defined based on a positive serological test for syphilis but a negative CSF test and no neurological symptoms or classic syphilis symptoms. Non-syphilis patients were negative based on serological and CSF tests.

2.2 Sample preparation

Approximately 1 mL of CSF was collected from each participant via lumbar puncture. The samples were transported on ice immediately after collection, and a syphilis serological test was carried out. CSF proteins and white blood cells were counted within six hours after CSF collection and stored at -80℃ until use. CSF samples were thawed, and 100 μL of sample and 300 μL of methanol were transferred to a 1.5-mL Eppendorf tube and vortexed for 30 s. All samples were kept at -40°C for 1 h, vortexed for 30 s and centrifuged at 12000 rpm and 4°C for 15 mins. Next, 200 μL supernatant and 5 μL DL-o-chlorophenylalanine (internal reference, 140 μg/mL) were transferred to vials for HPLC-MS analysis.

2.3 UPLC-Q Exactive MS

The HPLC-MS analysis was performed on an Ultimate 3000 UPLC system combined with Q-Exactive Orbitrap-MS (Thermo, Waltham, MA, USA). The LC system is comprised of an ACQUITY UPLC HSS T3 (100×2.1mm 1.8 μm) with Ultimate 3000LC. The mobile phase was composed of solvent A (0.05% formic acid-water) and solvent B (acetonitrile) with gradient elution (1-1.5 min, 95-70% A; 1.5-9.5 min, 70-5% A; 9.5-13.5 min, 5% A; 13.5-13.6 min, 5-95% A, 13.6-16 min, 95% A). The flow rate of the mobile phase was 0.3 mL/min. The column temperature was maintained at 40°C, and the sample manager temperature was set at 4°C. Mass spectrometry parameters in ESI+ and ESI- mode are listed as follows: Heater Temp 300 °C; Sheath Gas Flow rate, 45 arb; Aux Gas Flow Rate, 15 arb; Sweep Gas Flow Rate, 1 arb; Capillary Temp, 350 °C; S-Lens RF Level, 30%.; spray voltage, 3.0 kV in ESI+ mode and 3.2 kV in ESI- mode. The settings for full scan data acquisition were as follows: resolution, 70,000 fwhm; automatic gain control (AGC) target, 3×106; maximum injection time, 100 ms; scan range, 70–1050 m/z; polarity, negative or positive; spectrum data type, centroid. Ten quality control (QC) samples were run to avoid small changes in both chromatographic retention time and signal intensity at the beginning of the sequence. QC samples were also injected at regular intervals (every ten samples) throughout the analytical run [14].

2.4 MS data processing and identification

Raw data were acquired and aligned using Compound Discover (version 3.0, ThermoFisher Scientific, Waltham, MA, USA) according to the m/z value and the retention time of the ion signals. In the case of some large or small variables, normalization was often performed after alignment, and a line plot was used to evaluate the methodology. The confidence interval of 95% of the sample value was considered to be stable and feasible.

Normalized data were imported into the SIMCA-P program (version 14.1, Umetric, Umea, Sweden) for principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and the calculation of variable importance in the projection (VIP). As an unsupervised pattern recognition method for processing metabonomic data, PCA can classify metabolic phenotypes according to all input samples. All data from differentially expressed compounds were used to build PCA models. Different colours and icons represent different groups. PLS-DA was employed to supervise regression modelling of the data set to identify potential biomarkers. The quality of the models was described by relevant R2 and Q2 values. R2 displays the variance explained in the model and indicated the quality of the fit. Q2, as calculated by a cross-validation procedure, indicates the predictability of the model.

Fold change (FC) analysis and independent-sample t-test statistics were first applied for comparison of metabolite levels to determine statistically significant differences among the four groups. The cut-off criteria for screening differentially expressed metabolites were FC>2, VIP>1.5, and P<0.05. According to databases, the chemical structures of important metabolites were then identified, such as the Human Metabolome Database (http://www.hmdb.ca), using accurate mass and MS/MS fragment data.

HCA was performed and visualized by using the embedded module of MetaboAnalyst 4.0 [15]. By applying the Euclidean distance measure and Ward clustering algorithm, dynamic changes in significantly different metabolites were compared, and the ratio obtained was used to draw an HCA heat map. Colours in the heat maps correlate with the degree of increase (red) and decrease (blue) relative to the mean metabolite ratio.

Based on the differentially expressed metabolites, metabolic pathway and metabolite biofunction analyses were performed using the network database (KEGG pathway http://www.genome.jp/kegg/) to investigate the bioprocesses affected by *T. pallidum* infection. In brief, the enrichment level was calculated by the t-test, and metabolic pathways with P values less than 0.05 were considered statistically significant.

3. Results

3.1 Clinical information for the study participants

In addition to seven non-syphilis patients, 42 patients with neurosyphilis and 39 patients with latent syphilis were recruited in this study, including nine asymptomatic neurosyphilis patients. Because CSF samples from healthy people are challenging to obtain, samples from seven control groups were obtained, namely, from neurologic patients who were T. pallidum negative. The clinical characteristics of the patients are summarized in Table 1.

3.2 Quality control and overall metabolic profile

Data were subjected to a data integrity check before subsequent analysis, and no missing values were detected. The relative standard deviation (RSD) calculated from the ion features of the QC samples showed RSD values mostly less than 30%, indicating that the analysis program was reliable and could be used for subsequent sample analysis. The %RSD distribution is presented in Fig.1(a, b). The base peak intensity (BPI) chromatograms of the sample are illustrated in Fig.1(c, d). Among samples, 3799 and 3937 features were obtained in ESI+ and ESI- modes, respectively.

PCA was carried out using the molecular features of all the groups, and the distribution of metabolic profiles for the test samples in PCA is shown in Fig.2(a). The samples in each group were tightly clustered, but the difference between the groups was not large enough for a clear distinction. To eliminate any nonspecific effects of the technique and confirm the biomarkers for differentiating the metabolite profiles of non-syphilis controls and other syphilis patients (including latent syphilis and neurosyphilis

patients), a supervised PLS-DA model was established focused on the actual class discriminating variation. As depicted in Fig.2(b), due to the individual differences of the control samples, the distribution in the larger picture was scattered, but it was separated from the patient samples, with a clear distinction; R2Y was calculated to be 0.986 and Q2Y 0.632, which are greater than 0.4. To investigate dynamic changes in CSF metabolic profiles from syphilis to neurosyphilis, metabolomic data for the 39 latent syphilis and 42 neurosyphilis samples were analyzed by PLS-DA. As shown in Fig.2(c), we observed a clear separation between the two groups. Specifically, R2 and Q2 were 0.992 and 0.873 for differentiating syphilis patients and neurosyphilis controls, respectively. In addition to the obvious clinical manifestations, there was a significant difference in the metabolic spectrum between asymptomatic neurosyphilis and symptomatic neurosyphilis. According to the PLS-DA results (Fig.2d), there was a significant separation between the two groups with Q2Y=0.818 and R2Y=0.995.

3.3 Identification of differential CSF metabolites and pathway analysis

Based on the database search, 272 metabolites were identified in ESI- mode and 252 in ESI+ mode. Metabolites with VIP greater than 1.5 were included in t-tests to assess significant differences in CSF metabolites between different patients. A total of 20 metabolites obtained from the metabolic spectrum in both modes were filtered out compared to syphilis and non-syphilis patients, including ascorbic acid, acetaminophen, and fucose, which play an essential role in the immune system or nervous system. Next, five significantly different metabolites, nicotine, trihydroxycoprostanoic acid, 4-hydroxybenzoic acid (4-HBA), 5-hydroxy-L-tryptophan (5-HTP), and acetyl-N-formyl-5-methoxykynurenamine (AFMK), were found between patients with latent syphilis and patients with neurosyphilis. However, only theophylline showed a significant difference in comparison of the metabolic profiles of symptomatic neurosyphilis and asymptomatic neurosyphilis. Differences in expression of 5-HTP and AFMK between the two types of patients were also found, with no significant differences ($0.05<P<0.1$). Levels of both metabolites were up-regulated more than three times in the samples from patients with symptomatic neurosyphilis. It is worth noting that the level of 5-HTP differed between the non-syphilis control group and latent syphilis, symptomatic neurosyphilis, and asymptomatic neurosyphilis groups. The significant differential metabolite components are described in Table 2. According to the differential metabolites screened, a heat map of HCA was generated based on Euclidean distance and the average clustering algorithm, as shown in Fig.3.

Next, we performed a KEGG analysis of the differentially expressed metabolites to examine the effect of *T. pallidum* infection on metabolic pathways. The results showed that these differential metabolites were enriched in amino acids and metabolic intermediates of various acids. The metabolic pathways of enrichment are mainly related to amino acid metabolism, energy metabolism, and phosphatidic acid metabolism, such as the tricarboxylic acid cycle. Fig.4 shows that there were marginally significant differences ($P<0.1$) between the groups: the citric acid cycle, gluconeogenesis, tryptophan metabolism, ubiquinone biosynthesis, and caffeine metabolism. In particular, tryptophan metabolism was significantly different ($P<0.01$) between neurosyphilis and latent syphilis and between symptomatic neurosyphilis and asymptomatic neurosyphilis.

4. Discussion

In both latent syphilis and neurosyphilis, there are no apparent symptoms at the initial stage of infection, and serological tests are prone to false-positive as well as false-negative results, making syphilis diagnosis a clinical challenge. *T. pallidum* invades the nervous system during early primary syphilis, causing problems for the daily life of patients [16,17]. Therefore, it is imperative to screen, diagnose and treat suspected cases in a timely manner. Previously, Yang's team conducted a metabolomic analysis of serum and CSF samples from patients with syphilis and neurosyphilis [18]. Several significantly expressed metabolites, i.e., L-gulono-gamma-lactone, D-mannose, *N*-acetyl-L-tyrosine, and hypoxanthine, were identified in CSF and trimethylamine N-oxide in serum from neurosyphilis patients. In this study, a more detailed metabolomic analysis was carried out on 88 cerebrospinal fluid samples from patients with neurosyphilis (symptomatic and asymptomatic) and latent syphilis as well as those without syphilis. The metabolic analysis detected more than 400 metabolites, of which 20 were significantly different between syphilis and non-syphilis patients and five between neurosyphilis and latent syphilis patients. Only theophylline was significantly different between symptomatic and asymptomatic neurosyphilis patients. KEGG pathway enrichment analysis revealed that tryptophan metabolism pathways were significantly enriched, indicating a high correlation between tryptophan metabolism and syphilis symptoms.

Significant differences in metabolites and pathways were found between the syphilis group and the non-syphilis control group. The levels of 9,10,13-TriHOME, 9-OxoODE, PS (16:0/14:0), azelaic acid, and PS (16:0/14:0) were found to up-regulated in the syphilis group, but 2-HB and GDP were significantly down-regulated. Among the up-regulated metabolites, 9,10,13-TriHOME, 9-OxoODE, and PS (16:0/14:0) are lipid metabolites. PS is an active substance on the cell membrane, especially in brain cells, and its main function is to improve the function of nerve cells, sooth vascular smooth muscle cells and increase blood supply to the brain [19]. It has been confirmed that the membrane protein of T. pallidum contains phosphatidylserine lipoprotein, which may explain the increase in PS [20]. This might distinguish syphilis from false-positive VDRL caused by antiphospholipid syndrome due to antiphospholipid antibodies. In general, 9,10,13-TriHOME and 9-OxoODE are related to the oxidative metabolism of linoleic acid [21,22]. TriHOMEs are the end product of linoleic acid oxidation, which have a physiological role in maintaining the water-skin barrier, though other physiological effects are unclear [23]. Linoleic acid has a neuroprotective effect, and its deficiency has been found in patients with mild neurological disorders and Alzheimer's disease [24]. This may also explain the increase in linoleic acid metabolites in syphilis patients. Downregulated 2-HB is associated with dyslipidemia, involved in glutathione oxidative stress, and elevated in patients with depression [25].

Significant differences in 5-HTP and AFMK were found in neurosyphilis patients compared with latent syphilis, and it may constitute a marker of whether T. pallidum has invaded the nervous system. In some lymph node tumour cells, expression of indoleamine-2,3-dioxygenase (IDO1) is increased by activating regulatory T cells (Tregs) that escape the host immune system [26,27]. This enzyme is involved in the breakdown of indoleamine (tryptophan, 5-hydroxytryptamine, and melatonin). AFMK participates in the

kynurenine pathway, one of the three main metabolic pathways of melatonin and has potent antioxidant and anti-inflammatory abilities [28]. The high levels of AFMK observed in this study may result from melatonin production by local immune cells. 5-HTP is decarboxylated by aromatic L-amino acid decarboxylase with vitamin B6 as a cofactor to form serotonin (5-hydroxytryptophan, the precursor of melatonin). 5-HTP contributes to serotonin production, promoting emotional and nervous system health. Both melatonin and serotonin are important metabolites in regulating emotion. It has been suggested that the tryptophan-kynurenine pathway may be involved in depression by mediating the inflammatory response [25]. This may explain why some symptoms of neurosyphilis are consistent with depression; it also suggests that more attention should be paid to the mental state and emotional counselling of patients during treatment for neurosyphilis.

Only one metabolite was found to differ between symptomatic and asymptomatic neurosyphilis patients, which may be due to the different organs involved in symptomatic neurosyphilis. Theophylline is a metabolite of caffeine that can increase CSF secretion to regulate intracranial pressure and relieve headaches [29,30]. Theophylline also reduces smooth muscle tension, promotes endogenous epinephrine and norepinephrine release, inhibits calcium release from the endoplasmic reticulum of smooth muscle, and decreases intracellular calcium concentrations for respiratory tract dilation [31-33].

Due to the difficulty of lumbar puncture and poor compliance of patients, it is challenging to obtain CSF samples. Besides, this study enrolled few control samples. Overall, only preliminary conclusions were obtained in this study, and the sample size needs to be expanded for follow-up research. Nonetheless, some interesting conclusions were obtained. In the comparison between syphilis patients and non-syphilis patients, significant differences in lipids and derivatives were detected, indicating that lipid metabolism plays a vital role in syphilis. Thus, liposomics may be used as a further research direction. As neurosyphilis is usually the result of the late development of untreated syphilis, it is important to promptly distinguish neurosyphilis from latent syphilis. Both 5-HTP and AFMK are important metabolites for maintaining the health of the nervous system. Although differences in other comparisons were found for 5-HTP, they were not significant. The findings suggest that more attention should be paid to the mental state and emotional counselling of patients under treatment for neurosyphilis. The metabolites identified should be further studied to determine their relationship with T. pallidum infection.

In conclusion, we performed a metabolomics analysis of CSF samples from patients with symptomatic neurosyphilis, asymptomatic neurosyphilis, and latent syphilis. Through comparative analysis, we found that levels of linoleic acid metabolites were up-regulated in patients with syphilis. 5-HTP and AFMK in the tryptophan metabolism pathway were also significantly altered in neurosyphilis. Theophylline levels were significantly up-regulated in patients with symptomatic neurosyphilis. The role of these differential metabolites in the development of syphilis is worthy of further exploration. This analysis may facilitate the development of biomarkers for syphilis and determine the underlying molecular mechanisms associated with neurosyphilis.

Declarations

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Conflict of interest statement

No potential conflict of interest was reported by the authors.

Availability of data and material

Metabolomic data have been deposited to PeptideAtlas, and will be accessible under http://www.peptideatlas.org/PASS/PASS01659.

Code availability

Not available.

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College. Informed consents were obtained from all subjects. All experiments were performed following the approved guidelines.

Consent for publication

Not available.

Author contribution statement

JD and LL performed the experiments and carried out data analysis. XD, FC, and SC designed experimental procedures. JD and JZ conceived the study and wrote the manuscript. JZ, QJ and WL supervised this study. All authors read and approved the submitted version.

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Tables

Table 1. Clinical information for the study participants

Variable	asymptomatic neurosyphilis patients (W)	symptomatic neurosyphilis patients (Z)	latent syphilis patients (Y)	non-syphilis patients (F)	All
n	9	33	39	7	88
Age (Years)	42.89[47]	48.39[49]	35.49[32]	46.57[47]	41.97[42.5]
Sex					
Male n (%)	3(33.33%)	24(72.73%)	26(66.67%)	4(57.14%)	44(50.00%)
Female n (%)	6(66.67%)	9(27.27%)	13(33.33%)	3(42.86%)	44(50.00%)
Serological test	+	+	+	-	
CSF TRUSRT	+	+	-	-	
Clinical symptoms	No obvious neurological symptoms	Meningitis, stroke, acute changes in mental status or abnormal hearing and vision	No obvious clinical manifestations	-	

CSF TRUSRT, cerebrospinal fluid toluidine red unheated serum test.

Table 2. List of differential CSF metabolites in comparisons of different study groups

Comparison	Metabolite	RT [min]	Monoisotopic Mass	VIP	FC	P-value
syphilis vs. non-syphilis	2-Hydroxybutyric acid	1.626	104.046	2.881	0.490	<0.001
	Azelaic acid	2.739	188.104	1.535	2.020	0.027
	9-OxoODE	4.994	294.219	1.897	4.422	0.009
	9,10,13-TriHOME	5.026	330.241	2.222	5.125	0.001
	Guanosine diphosphate	1.579	443.012	2.340	0.105	0.001
	PS (16:0/14:0)	4.116	707.466	1.763	3.049	0.015
latent syphilis vs. neurosyphilis	4-Hydroxybenzoic acid	2.323	138.032	2.039	4.177	<0.001
	5-Hydroxy-L-tryptophan	1.518	220.084	1.797	4.674	0.000
	Acetyl-*N*-formyl-5-methoxykynurenamine	2.320	264.111	1.850	3.882	0.000
	Nicotine	0.822	162.115	2.037	2.927	<0.001
	Trihydroxycoprostanoic acid	10.682	464.361	2.298	2.672	<0.001
Asymptomatic vs. symptomatic neurosyphilis	Theophylline	1.918	180.065	3.091	9.718	0.001

RT for retention time; VIP for variable importance plot; FC for fold change.

Figures

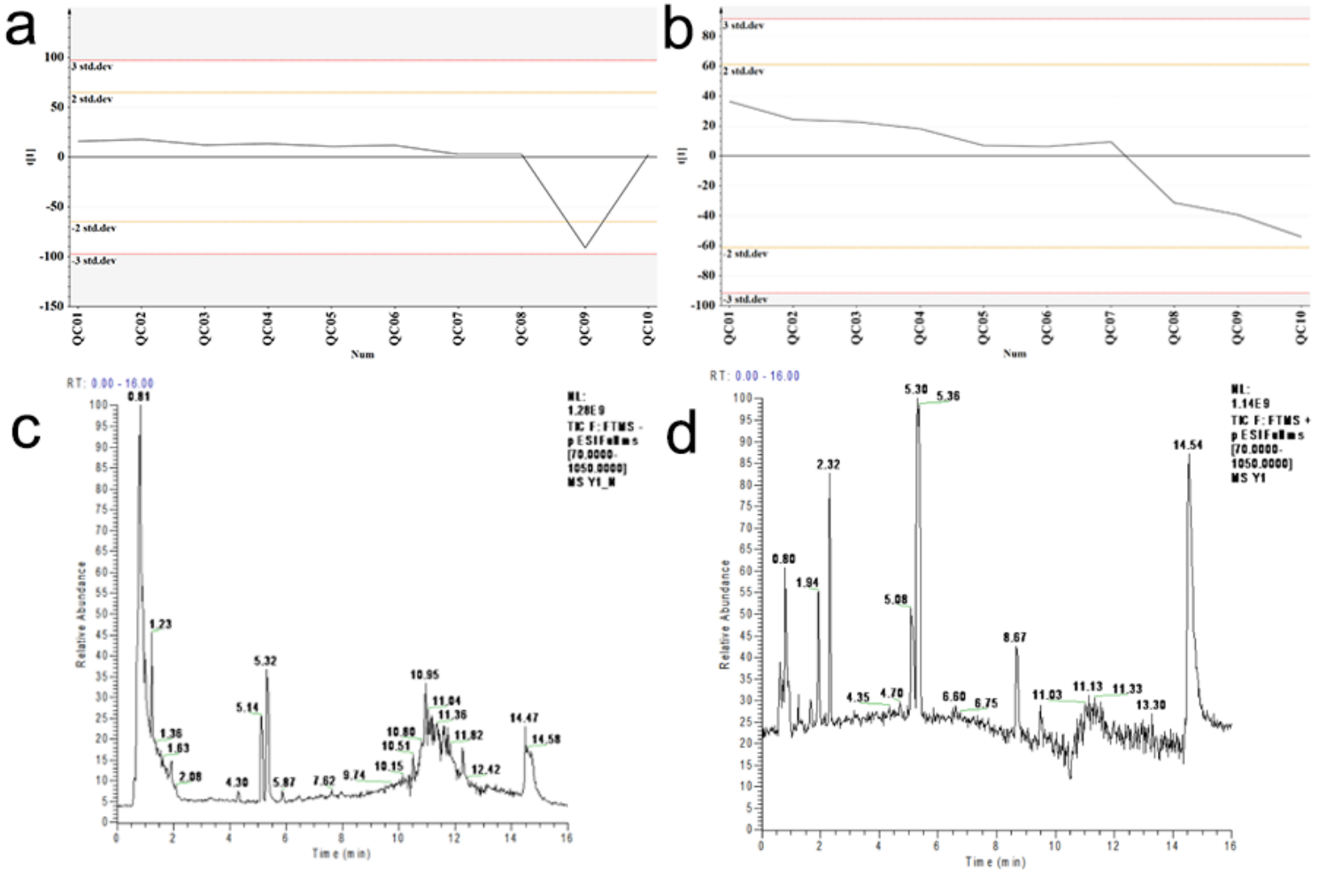

Figure 1

(a, b), the score plot of the quality control (QC) samples, the X-axis indicates the number of QC samples; the Y-axis indicates the range of relative standard deviation (RSD). (c, d), base peak intensity (BPI) chromatograms of the sample. (a, c) in ESI- mode, and (b, d) in ESI+ mode.

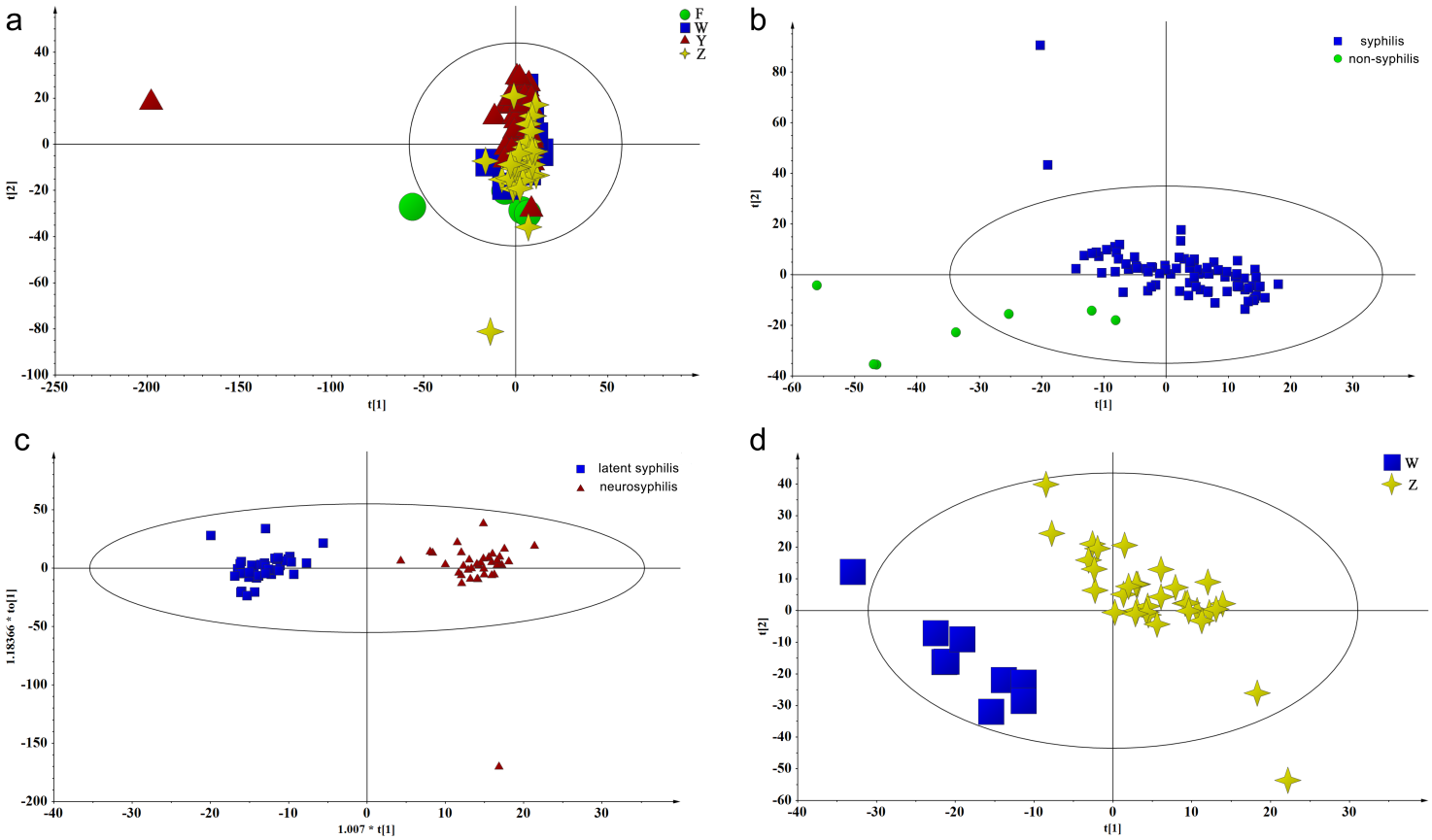

Figure 2

(a), the scores scatter plot of principal components analysis (PCA) model, R2X=0.422. (b-d), The scores scatter plot of partial least squares discriminant analysis (PLS-DA) model. (b), the PLS-DA model based on non-syphilis controls and other syphilis patients;(c), the PLS-DA model based on latent syphilis and neurosyphilis patients;(d), the PLS-DA model based on asymptomatic and symptomatic neurosyphilis patients.

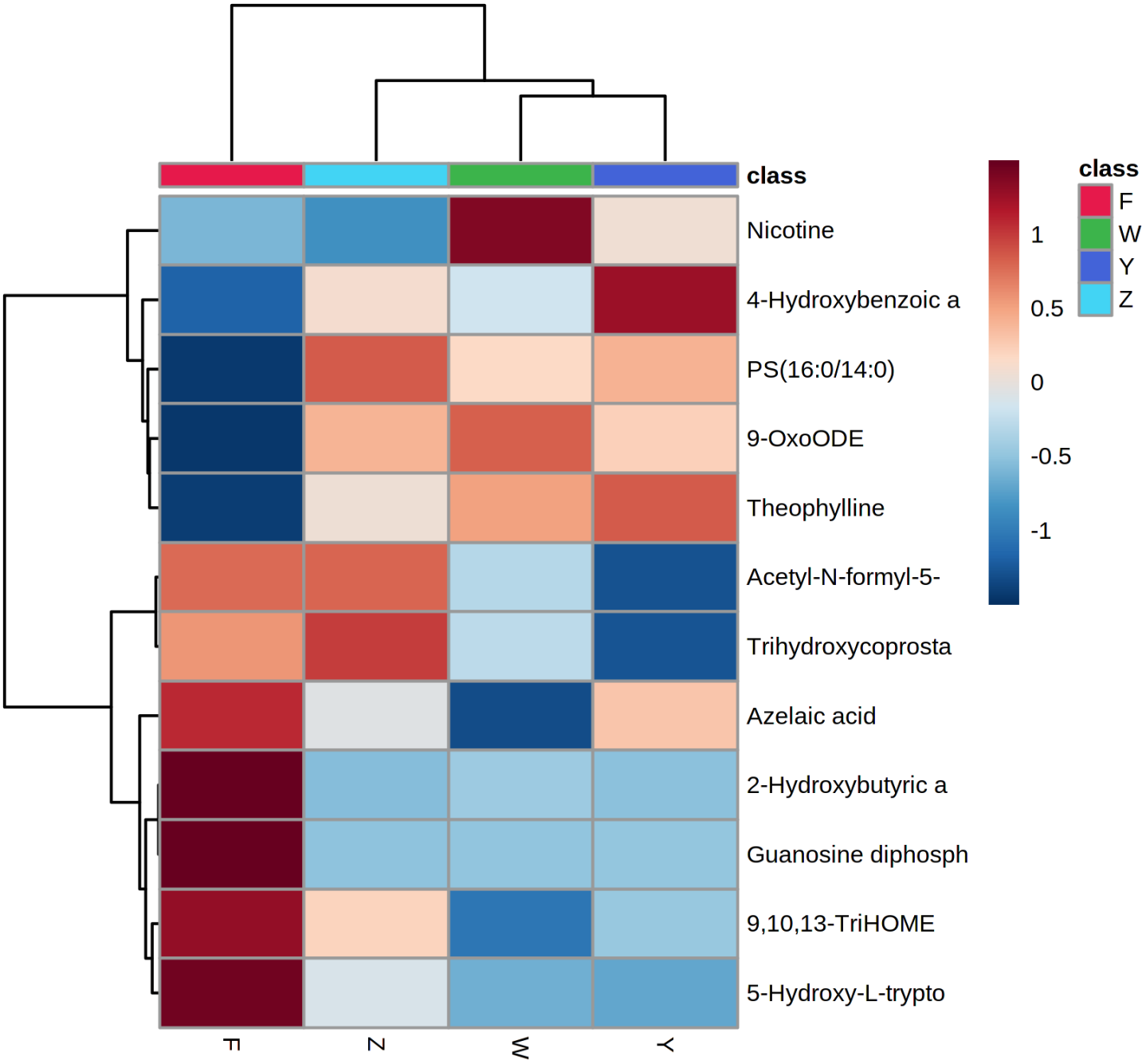

Figure 3

Heat map based on differential metabolites of latent syphilis (Y), asymptomatic neurosyphilis (W), symptomatic neurosyphilis (Z) and non-syphilis control (F).

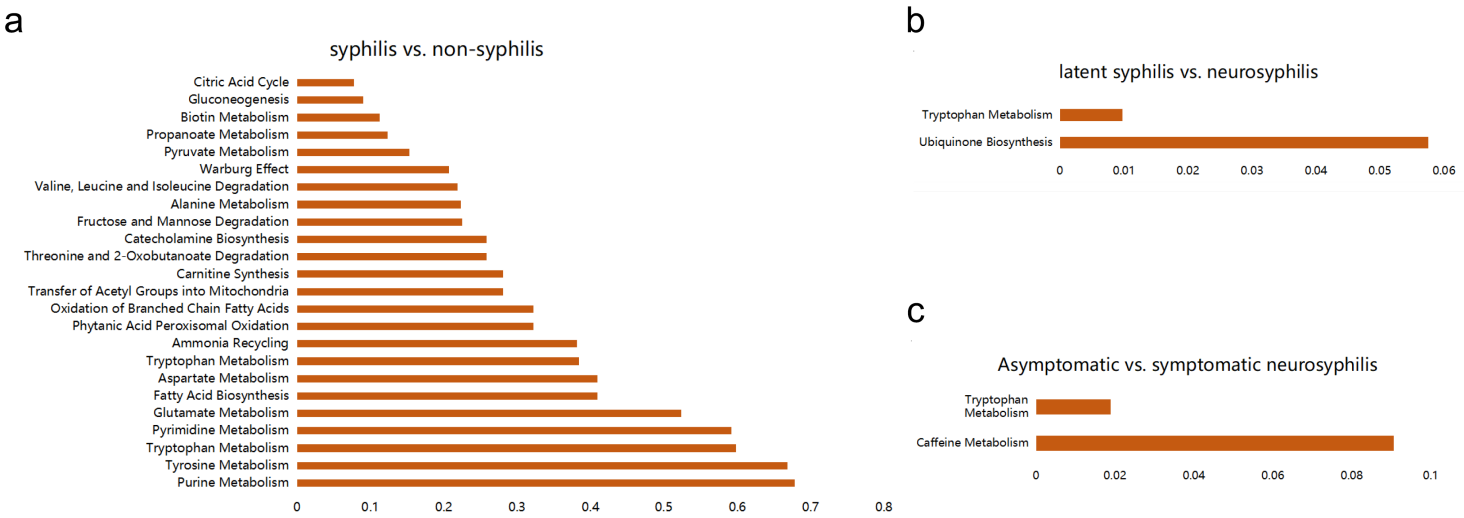

Figure 4

The bar chart of enriched KEGG pathways of the differential metabolite components in the comparisons. The X-axis indicates the P-value of each pathway; Y-axis indicates the name of the pathway.