Original Article

1H NMR Using Metabolic Study in Body Fluids for Diagnosis of Cryptococcal Meningitis in Adults

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Abstract

Background: Cryptococcal meningitis is considered to affect HIV patients and those with impaired immune systems. Early identification and treatment are the keys to decreasing morbidity and mortality related to CM. Using 1H NMR spectroscopy, a prospective case–control study will assess the metabolic profile of adults' serum, urine, and CSF. **Methodology:** The present multicentric study was conducted at Lucknow. The study included 150 participants, out of which there were 31 cryptococcal meningitis cases, 34 positive meningitis controls, and the rest, 85, were disease controls. **Result:** The discriminant function analysis (DFA) of the three biofluids was used to find significant metabolites between the cases and the control group collectively. A group categorization between control group and the cases in serum, urine, and CSF samples was also made possible by the NMR spectral bin-based orthogonal signal correction and principal component analysis score plots of important metabolites produced from DFA. The cases group had a higher proportion of patients with higher CSF protein levels than the positive control group (BM and TM). Acetone was found among urine samples in both control samples, i.e., positive and negative. **Conclusion:** This is the first study to explore biomarkers in serum, urine, and CSF in addition to radiological features and clinical symptoms. Hence, a quick, non-invasive prognosis and diagnosis of cryptococcal meningitis in adults can be made using clinical and microbiological investigation, as well as metabolomic analysis of urine samples. This study shows that urine can be used as a biofluid to differentiate between Cryptococcus meningitis in adults. However, when compared to the negative control, our sample size was significantly smaller, necessitating further confirmation on a larger sample size.

Keywords: Cerebrospinal fluid, HIV, NMR spectroscopy cryptococcal meningitis, serum, urine

Introduction

The meninges, which are the protective membranes that surround the brain and spinal cord, become inflamed when someone has meningitis.[1] Inflammation may be caused by viruses, bacteria, fungus, or other organisms, as well as less frequently by some drugs.[2] The proximity of the inflammation to the brain and spinal cord makes meningitis a medical emergency, which makes it potentially fatal.[3]

Cryptococcus neoforms, an encapsulated yeast, are adaptable fungal pathogens of the central nervous system (CNS) that can cause cryptococcal meningitis, a potentially fatal infection that commonly affects immunocompromised individuals.[1,2] Among AIDS patients, CM is the fourth most frequently identified cause of a life-threatening infection, with an estimated 223,100 annual incidence and more than 1,81,000 annual deaths worldwide.[2]

Despite the fact that many radiological investigations of cryptococcal meningitis are helpful, probably the most significant analytical methods for obtaining metabolic information from biological fluids or tissue extracts in recent years have been high-resolution proton NMR spectroscopy.[4,5] Previously, substantial NMR spectroscopy research on the metabolite composition of normal CSF revealed that it contains a relatively small range of metabolites that are quite stable with diet and medicine and can only be stored at room temperature for a short time.[6,7] A metabolomic technique based on NMR spectra from CSF has been established in a different study utilizing an animal model to determine whether metabolites produced by an infection-causing bacteria or a particular immune response can be used for meningitis diagnosis.[8,9]

It is vital to conduct research to determine the superiority of proton NMR spectroscopy in order to offer an evidence-based, well-informed clinical recommendation. As a result, the purpose of this study was to evaluate NMR spectroscopy in conjunction with metabolic studies in bodily fluids for the diagnosis of cryptococcal meningitis.

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Materials and Methods

The present observational and analytical case–control study was conducted at the Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, and the Ethical approval was taken by Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow (IEC No. 6/14) on dated 2/06/2014. The inclusion criteria considered for the present study were patients aged 18 years and older and cases complying with the case definition of cryptococcal meningitis. Exclusion criteria included participants under the age of 18, patients for whom lumbar puncture was contraindicated, and a patient or representative attendant of a patient who did not give consent.

Procedure

The study enrolled a total of 150 patients who met the inclusion criterion during the study period. For diagnostic, therapeutic, and prognostic purposes in suspected cases of subacute and chronic meningitis, as well as in diseased controls, all additional CSF samples received for microbiological and pathological evaluation from the Neurology Department of Dr. RMLIMS/KGMU were tested for Cryptococcus. The cases with the positive test were included in the study. Leftover CSF from these cases was stored by snap-freezing or in liquid nitrogen and sent for NMR evaluation. A detailed history and clinical evaluation of the patient were done. Wherever possible, a correlation with radiological investigations was attempted.

A blood sample was also collected from the patient for estimation of the corresponding blood glucose level as per standard protocol. Blood was collected in a plain vial and urine in a sterile container within 24 hours of admission. A record of the types of drugs administered to the patient for metabolomic correlation was kept.

The leftover CSF sample from the suspected cases of subacute and chronic meningitis was used to perform the cryptococcal latex agglutination test (LAT) with CALAS (Bio-Rad, PastorexTM Crypto Plus, or any other equivalent manufacturer's kit), as well as a wet mount examination, fungal culture, and fungal growth on Sabouraud dextrose agar (SDA).

Apart from testing for Cryptococcus, the CSF was also evaluated for other bacterial pathogens by bacterial culture, Gram's and Ziehl–Neelsen staining, which was done on chocolate agar, blood agar, and MacConkey agar, and inoculation in brain heart infusion broth (BHI), including Mycobacteria, as per standard techniques, including use of Lowenstein–Jensen media. Isolates were identified using the standard protocol if culture results were determined to be positive. Urine was also cultured for bacterial pathogens for metabolomic correlation. Culture-positive urine samples

Table 1: Groupwise distribution

Group	No. of patients	Percentage
Disease control	85	56.67%
Positive control (BM + TBM)	34	22.67%
Cases (CM)	31	20.67%
Total	150	100

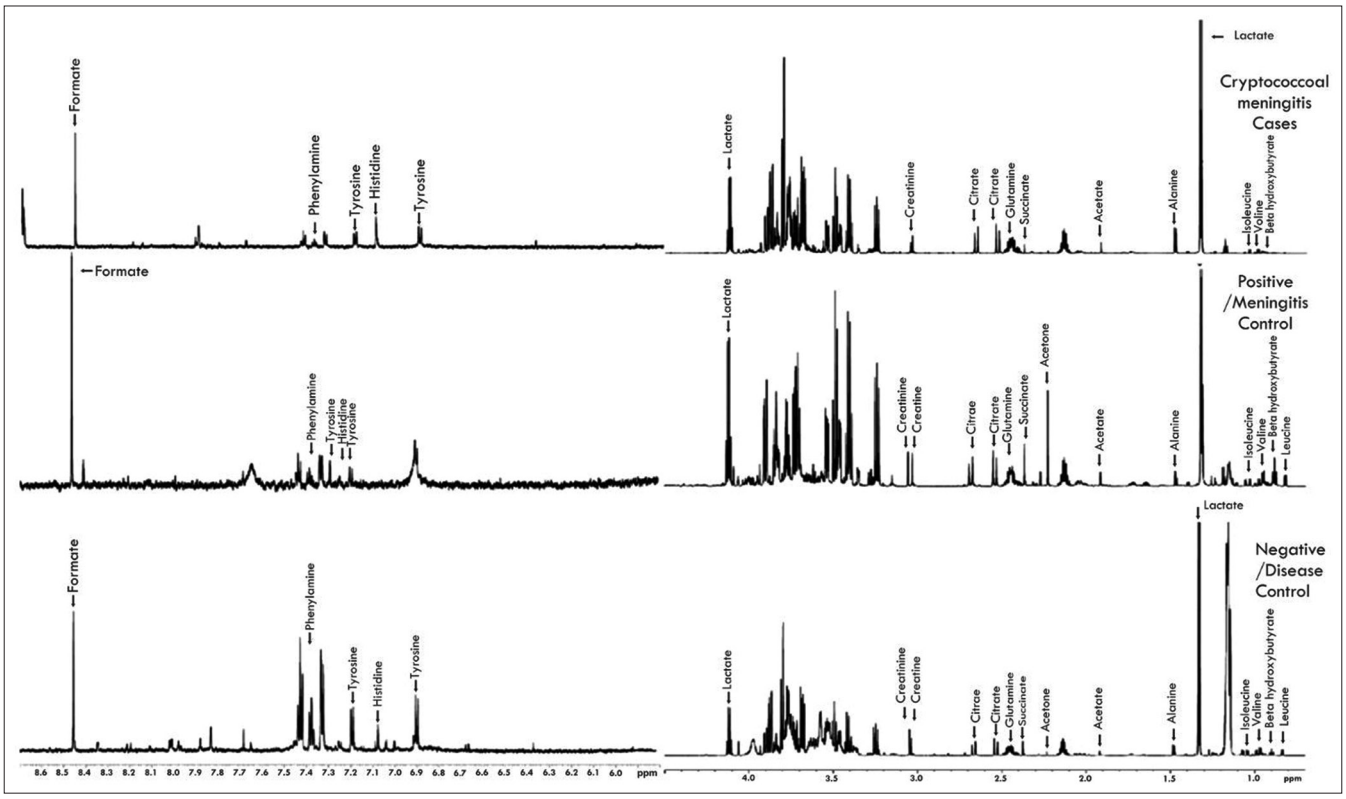

Figure 1.1: NMR spectra of CSF samples

from cases or controls were excluded from metabolomics analysis.

Metabolomic analysis

1 ml of the CSF, serum, and urine were snap-frozen in liquid nitrogen (-80°C) after collection and sent to the Centre for Biomedical Research (CBMR), SGPGIMS, Lucknow, for metabolomic analysis. For comparison of NMR spectra of CSF, serum, and urine with meningitis cases and diseased controls, positive controls were processed. The NMR investigations were carried out using an Avance III 800 MHz FT-NMR spectrometer from Bruker (Fallanden, Switzerland) that operates at a proton frequency of 800.26 MHz and is outfitted with a 5 mm triple resonance 1H/13C/15N TCI cryoprobe with a Z-shielded gradient.

Data analysis

Clinical symptom percentages and the overall number of patients for whom a particular symptom was documented were both included in the data that were provided. Metabolites found in NMR spectra as well as pertinent clinical data, such as details on the CSF cell count and the CSF protein content, were taken into consideration for multivariate analysis in order to define significant descriptors for the differentiation of cryptococcal meningitis from the control group.

Result

According to Table 1, the largest proportion of cases was observed in the disease control group, with 85 cases representing 56.67% of the total cases. The positive control group had 34 cases, which accounted for 22.67% of the total cases, while the study group had 31 cases of CM, representing 20.67% of the total cases.

Among the positive control group, 13 patients (38.24%) were female, while 21 patients (61.76%) were male. Among the cases group, 7 patients (22.58%) were female, and 24 patients (77.42%) were male. However, the Chi-squared test

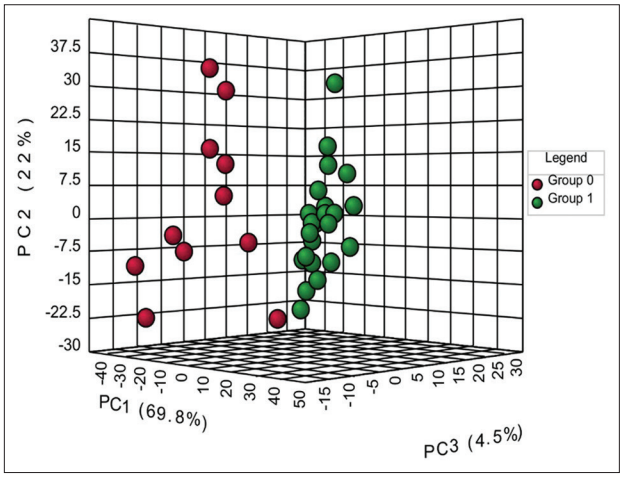

Figure 2.1: 3D OSC-PCA score plot based on CSF samples effectively separates the positive control and cases, indicating a significant difference between the two groups

for this comparison showed a non-significant result ($\chi 2 = 1.85$, $P = 0.17$), demonstrating that the gender distribution between the case group and the positive control group did not differ significantly.

Clinical Sign/Symptom: The positive control group had 7 patients (20.59%) with generalized convulsions, while the cases group had only 1 patient (3.23%) with this symptom. Evidence of Source of Infection: The positive control group had only 2 patients (5.88%), while the cases group had 14 patients (45.16%) with evidence of the source of infection. The result of the statistical test for this comparison is reported as <0.05 History of Antibiotic Intake: The positive control group had 13 patients (38.24%) with a history of antibiotic intake prior to admission, while the cases group had only 10 patients (32.26%) with this history. The result of the statistical test for this comparison is reported as $\chi 2 = 24.89$ and $P < 0.0001$.

CSF Biochemistry –The positive control group had a higher proportion of patients with higher CSF protein levels than the cases group. Specifically, the positive control group had 28 patients (82.35%) with CSF protein levels between 44 and 1000 mg%, while the cases group had only 31 patients (58.06%) in this category with statistically significance difference ($P = 0.006$). In the microbiological examination, all the variables show statistically significance difference between positive control with $P < 0.05$.

In the disease control group, 37 patients (43.53%) were female, and 48 patients (56.47%) were male. In contrast, the cases group had 7 female patients (22.58%) and 24 male patients (77.42%). However, the Chi-squared test used to compare these groups showed a non-significant result ($\chi 2 = 4.23$, $P = 0.003$), suggesting that there was no significant difference in the gender distribution between the disease control and cases groups.

Regarding clinical signs and symptoms, vomiting was reported in 24 patients (28.24%) in the disease control group and 22 patients (70.97%) in the cases group. Evidence of the source of infection was found in only 3 patients (3.53%) in the disease control group, while 14 patients (45.16%) in the cases group had such evidence. The statistical test showed that this difference was highly significant (<0.001).

In terms of CSF biochemistry, the disease control group had a higher proportion of patients with CSF protein levels between 44 and 1000 mg% (49.41%) than the cases group (48.39%). This difference was statistically significant (p = <0.001). In the microbiological examination, there was a statistically significant difference between the disease control and cases groups in India ink positive and Gram stain organism with a *P* value of less than 0.05 [Table 2].

Based on the presence of important metabolites, cases of BM and TBM in the positive control group can be distinguished in the three-dimensional dispersed plot created by OSC-PCA analysis. Blue and red circles and squares, respectively, stand in for these metabolites. As shown in Figure 2.1, the OSC-PCA

Table 2: Positive control and disease control vs. cryptococcal meningitis cases

Characteristic	Positive control ($n=34$)		Cases ($n=31$)		Statistical significance	
	No.	%	No.	%	χ^2	'*P*'
Gender						
Female	13	38.24	7	22.58	1.85	0.17
Male	21	61.76	24	77.42		
Mean (Age±SD)	37.5±19.9		42.61±17.93		*t*=1.08, 0.28	
Clinical Signs and Symptoms						
Fever	27	79.41	29	93.55	2.71	0.09
Headache	25	73.53	25	80.65	0.22	0.63
Convulsions—generalized	7	20.59	1	3.23	4.52	0.03
Convulsions—focal	1	2.94	2	6.45	0.45	0.50
Nausea/Vomiting	26	76.47	22	70.97	0.25	0.61
Focal weakness/Neurological deficit	4	11.76	1	3.23	1.65	0.19
Altered sensorium	5	14.71	12	38.71	2.24	0.13
Lymphadenopathy	0	0	0	0	-	-
Abnormal movements	3	8.82	3	9.68	0.01	0.90
Signs of meningeal irritation	5	14.71	1	3.23	2.55	0.11
Evidence of source of infection	2	5.88	14	45.16	13.48	0.002
Any other	14	41.18	11	35.48	0.22	0.63
H/o Antibiotic intake prior to admission						
No	1	2.94	18	58.06	28.08	<0.0001
Yes	13	38.24	10	32.26		
Unknown	20	58.82	3	9.68		
CSF Biochemistry						
CSF cell count/cm						
0-5	6	17.65	6	19.35	9.35	0.052
6-50	16	47.06	7	22.58		
50-100	4	11.76	11	35.48		
100-500	7	20.59	4	12.90		
>500	1	2.94	4	12.90		
CSF Protein (mg %)						
0-8	0	0	0	0	14.60	0.006
9-43	6	17.65	3	9.68		
44-200	27	79.41	15	48.39		
201-1000	1	2.94	13	41.94		
>1000	0	0	0			
Mean CSF sugar±SD	59.00±19.15		51.8±36.16		*t*=1.01, 0.31	
Mean RBS±SD	107.49±15.72		119.59±32.90		*t*=1.91, 0.059	
Microbiological examination						
WM pus cells	33	97.06	16	51.61	18.04	<0.001
WM organism	0	0	14	45.16	19.57	<0.001
India ink positive	0	0	24	77.42	41.73	<0.001
Gram stain pus cells	33	97.06	16	51.61	18.04	<0.001
Gram stain organism	0	0	18	58.06	27.30	<0.001
CALAS positivity	0	0	31	100	65.00	<0.001

score plot of CSF (cerebrospinal fluid) revealed that 74.3% of the entire variation in the data was explained.

As shown in Figure 2.2, the 3D-dispersed score plot derived from the OSC-PCA analysis of the CSF samples showed that 47.9% of the total variation in the data could be explained. Notably, the disease control group was observed to form a distinct cluster that was clearly separated from that of the cases [Figure 2.2].

The DFA resulted down-regulation in the levels of acetoacetate, citrate, glucose, and formate in individuals with meningitis, similarly disease control group, decrease in concentration of glucose and formate in cases group observed. By using multivariate DFA to distinguish between cases and the positive control, we achieved an accuracy of 91.1%, with 100% sensitivity and 84.6% specificity. In terms of differentiating between the disease group and the cases group, we obtained

an accuracy, sensitivity, and specificity of 94.1%, 95.6%, and 90.9%, respectively [Table 3 and Figure 1.1].

In the three-dimensional scattered plot generated by OSC-PCA analysis, cases of TBM and BM in the positive control group can be differentiated based on the presence of significant metabolites. Red circles and blue squares were used to denote these metabolites, respectively. The OSC-PCA score plot of serum showed that 46.7% of the total variance in the data was explained, as shown in Figure 4.1.

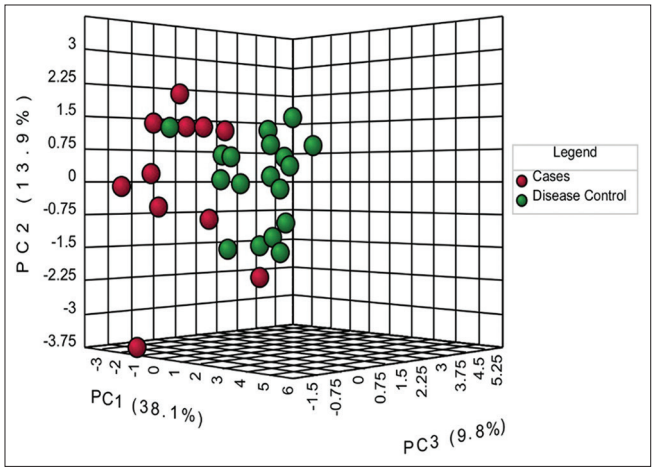

Figure 2.2: 3D OSC-PCA score plot based on CSF samples effectively separates the disease control and cases, indicating a significant difference between the two groups

By performing OSC-PCA analysis on serum samples, we were able to generate a 3D scattered score plot that explained 34.1% of the total variance in the data, as depicted in Figure 2.1. Remarkably, we observed that the disease control group formed a well-defined cluster that was distinctly separate from the cases [Figure 4.2].

The DFA led to a reduction in formate levels and an increase in acetate levels in meningitis patients. Additionally, the disease control group showed an increase in the concentrations of isoleucine, valine, acetate, and succinate in the cases group. When using multivariate DFA to differentiate between cases and the positive control group (BM and TBM), we achieved an accuracy of 73.1%, with a sensitivity of 72.2% and a specificity of 75%. Moreover, for distinguishing between the disease control and the cases group, we obtained an accuracy of 81.8%, a sensitivity of 78.5%, and a specificity of 90% [Table 3 and Figure 3.1].

By performing OSC-PCA analysis, we were able to create a scattered plot with three dimensions. This plot helped us differentiate between BM and TBM cases in the positive control group by identifying significant metabolites. Red circles and blue squares were used to denote these metabolites, respectively. Furthermore, the serum OSC-PCA score plot showed that 48.8% of the overall variability in the data was explained [Figure 6.1].

Through the application of OSC-PCA analysis on urine samples, we produced a 3D scattered score plot that accounted

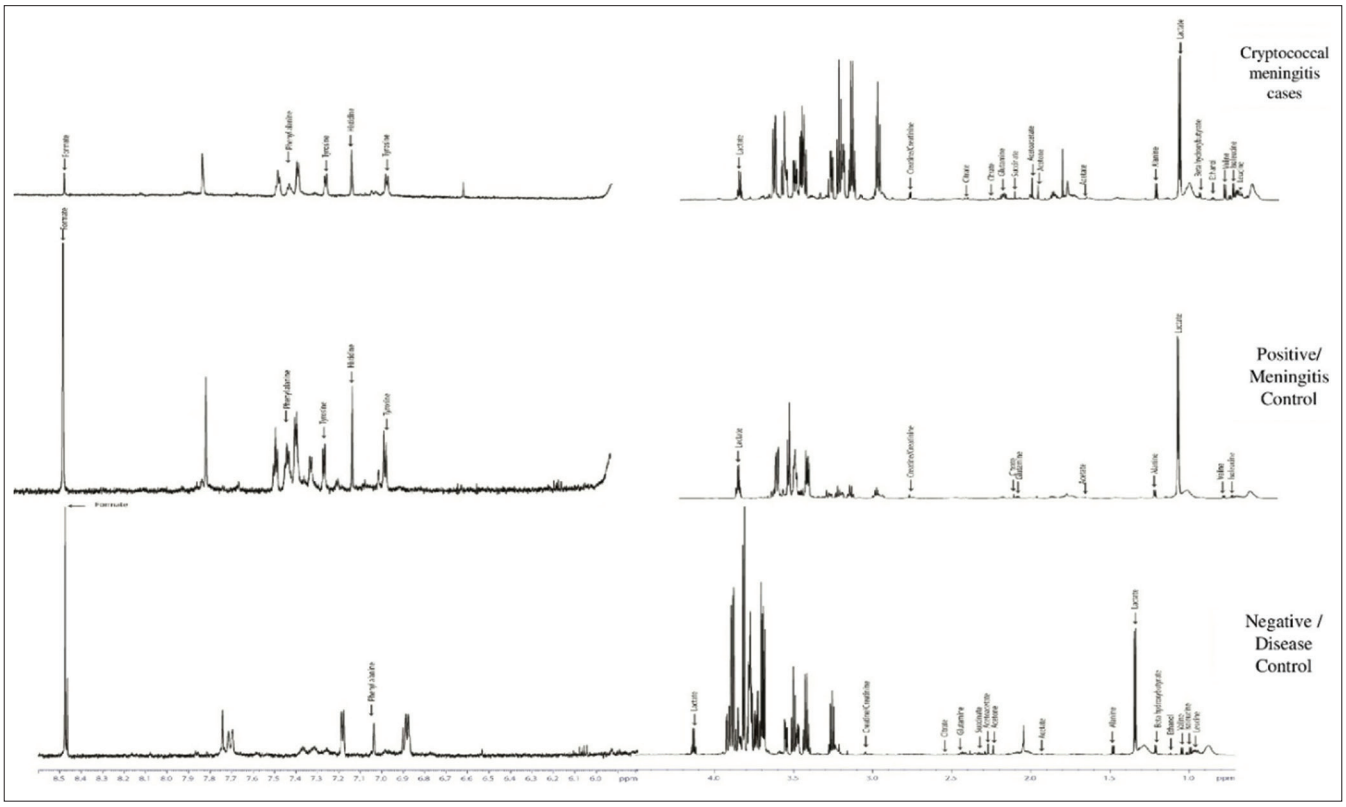

Figure 3.1: NMR spectra of serum samples

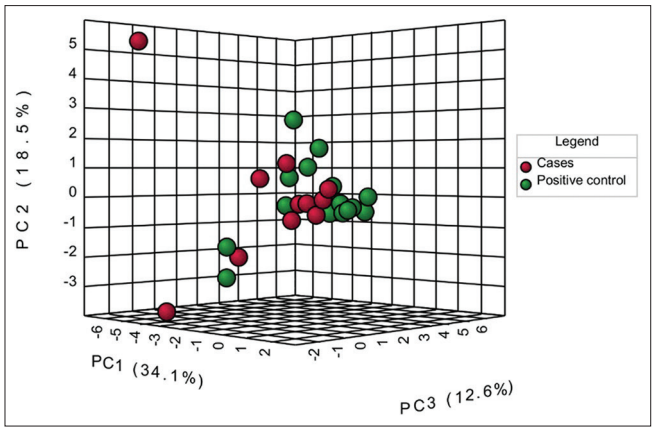

Figure 4.1: 3D OSC-PCA score plot based on serum samples effectively separates the positive control and cases, indicating a significant difference between the two groups

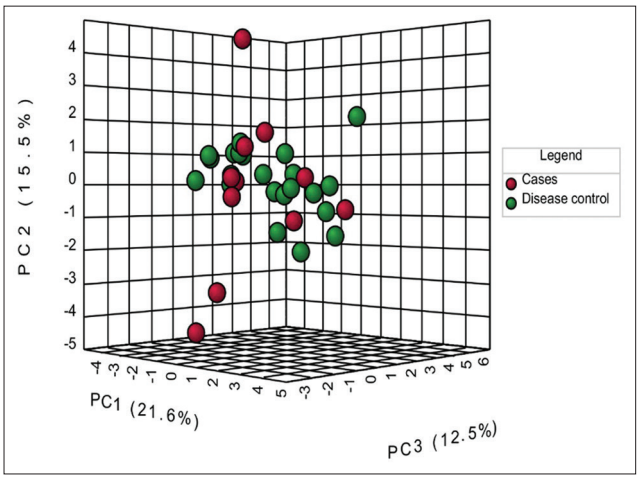

Figure 4.2: 3D OSC-PCA score plot based on serum samples effectively separates the disease control and cases, indicating a significant difference between the two groups

for 48.3% of the overall variance in the data, as illustrated in Figure 3.1. Our analysis revealed that the disease control group formed a distinct cluster that was somewhat segregated from the cases [Figure 6.2].

In both the positive control and disease control groups with cases, the DFA classification results revealed an up-regulation in the levels of acetone. When using multivariate DFA to distinguish between cases and the positive control group (BM and TBM), we obtained an accuracy of 80%, with a sensitivity of 79.1% and a specificity of 100%. Furthermore, for distinguishing between the disease control and the cases group, we achieved an accuracy of 72%, a sensitivity of 68.7%, and a specificity of 77.7% [Table 3 and Figure 5].

Discussion

The findings of the current prospective case–control study, which examined the metabolic profile in serum, urine, and CSF in adults using 1H NMR spectroscopy, a non-distractive method, demonstrated the ability of metabolomics for the quick, painless, and non-invasive diagnosis of CM.

The subsequent studies have shown that NMR metabolic profiling of biofluids is a highly effective and comprehensive method. It has been further discussed how to distinguish meningitis cases from disease controls in urine and serum samples because it could help to explain the pathology associated with meningitis by comparing meningitis cases to disease controls.

Metabolic profile of CSF

The metabolites that distinguished between meningitis cases and disease control using stepwise DFA were glucose and formate.

According to a study by:

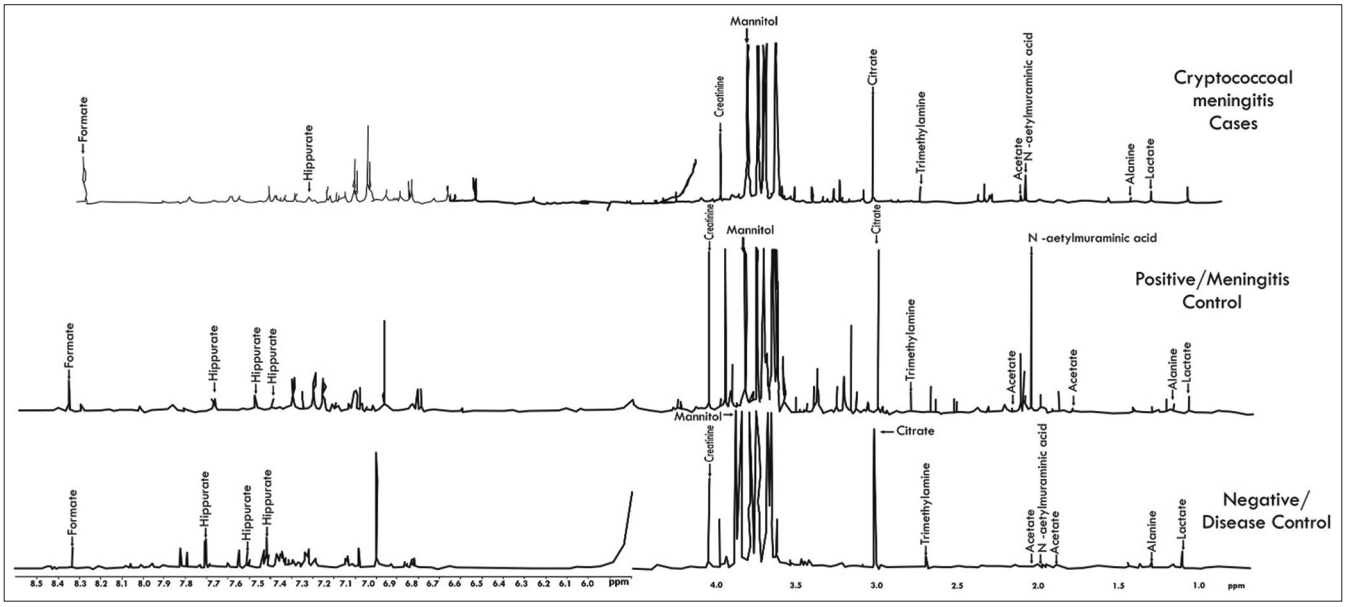

Figure 5: NMR spectra of urine samples

Table 3: Identification of significant metabolites in CSF, serum, and urine for discrimination between meningitis cases and disease controls, and between positive control groups using multivariate stepwise DFA

Groups	Significant Metabolites	Correct Classification (%)	Sensitivity (%)	Specificity (%)
CSF				
Positive Controls vs. Cases	Acetoacetate (↓ cases) Citrate (↓ cases) Glucose (↓ cases) Formate (↓ cases)	93.1	100	84.6
Disease Controls vs. Cases	Glucose (↓ cases) Formate (↓ cases)	94.1	95.6	90.9
Serum				
Positive Controls vs. Cases	Acetate (↑ cases) Formate (↓ cases)	73.1	72.2	75
Disease Controls vs. Cases	Isoleucine (↑ cases) Valine (↑ cases) Acetate (↑ cases) Succinate (↑ cases)	81.8	78.5	100
Urine				
Positive Controls vs. Cases	Acetone (↑ case)	80	79.1	100
Disease Controls vs. Cases	Acetone (↑ case)	72	68.7	77.7

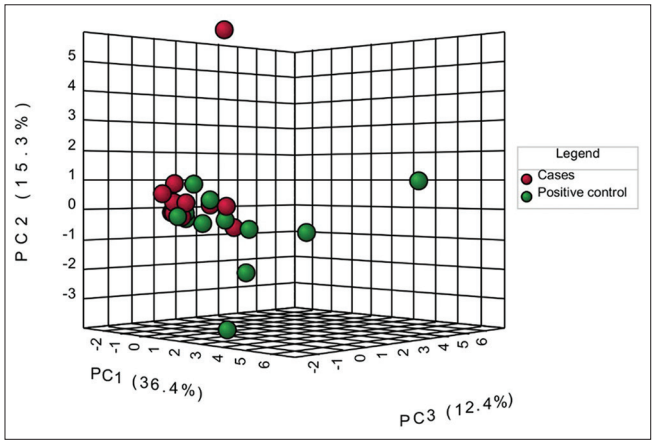

Figure 6.1: 3D OSC-PCA score plot based on urine samples effectively separates the positive control and cases, indicating a significant difference between the two groups

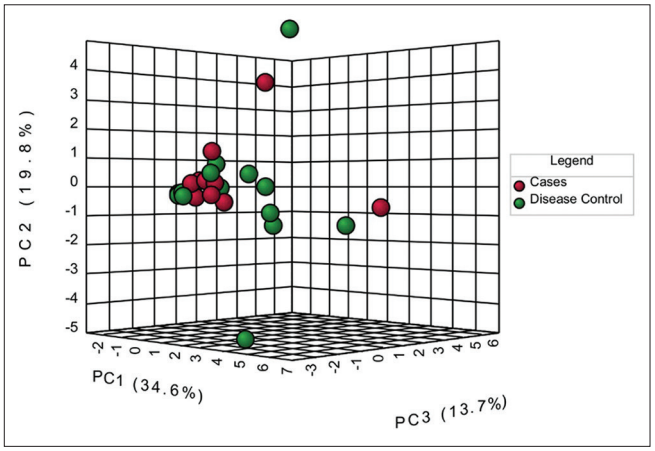

Figure 6.2: 3D OSC-PCA score plot based on urine samples effectively separates the disease control and cases, indicating a significant difference between the two groups

Coen *et al*.,[10] Patients with meningitis have a disproportionate up-regulation of lactate, according to numerous reports. The rise may result from increased glucose catabolism (through cerebral glycolysis brought on by ischemia and cytokine release), which produces pyruvate before converting it to lactate.

Lannigan *et al*.,[11] Huy *et al*.,[12] Prasad and Sahu,[13] and Sakushima *et al*.[14] It has been widely reported in the literature that meningitis causes a rise in lactate levels as well as the depletion of glucose by using a biochemical study of CSF. Thus, in our study, a substantial association between metabolomic and biochemical examinations was found.

Cunha,[15] Meningitis can be distinguished from other causes of reduced CSF glucose levels in adults using CSF lactate levels as a diagnostic tool to determine the cause of adults' lowered CSF glucose levels.

Metabolic profile of serum

When compared to both the disease control group and the meningitis case controls, the metabolite acetate was found to be considerably elevated in the serum samples of the patients. The intestinal bacterial fermentation of indigestible carbohydrates and, to a lesser extent, proteins was discovered to create acetate, a short-chain fatty acid, and other metabolites in the greatest levels.[7] It is the by-product of lipid metabolism and can be converted to acetyl-coenzyme A by the enzyme acetyl-CoA synthetase.[16]

According to Khovidhunkit *et al*.[17] The majority of the results of the Mann–Whitney U-test, DFA, and VIP score show that during meningitis, serum samples had higher levels of ketonic bodies, succinate, ethanol, alanine, lactate, and glucose. According to reports, both in humans and laboratory

animals, the content of plasma free fatty acids can change significantly (increase or decrease). Infection may be the cause of the increase in ketonic body levels.

Metabolic profile of urine

When compared with both the control group, acetone was the only metabolite which was found to be significantly elevated in the urine samples of meningitis cases. As a result of the CM group's lack of carbohydrates, energy is produced via the oxidation of fatty acids and ketone bodies, including acetone, which is made from acetyl-CoA. The rise in acetone metabolites seen in the urine of these instances may suggest that CM patients are using store lipids as an alternative energy source.[18]

Conclusion

This pilot study has established the viability of significant metabolites for the rapid diagnosis of cryptococcal meningitis using 1H NMR spectroscopy. This is the first study to explore biomarkers in serum, urine, and CSF in addition to radiological features and clinical symptoms. Thus, utilizing clinical and microbiological examination, a rapid, non-invasive prognosis and diagnosis of cryptococcal meningitis in adults can be made as well as metabolomic analysis of urine samples. The study shows that a high level of significance may be achieved when meningitis patients are distinguished from the control group (both disease control and positive control) using inclusion criteria, exploratory multivariate data modeling techniques, and high-field 1H NMR spectroscopy. This study shows that urine can be used as a biofluid to differentiate between Cryptococcus meningitis in adults. However, compared to the negative control, the sample size in our investigation was substantially smaller, necessitating further confirmation on a larger sample size.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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