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A Study of Clinical Spectrum, Laboratory Profile, Clinical Outcome and Comprehensive Metabolomic Analysis of Vivax Malaria in Admitted Children in PBM Hospital, Bikaner, North Western India

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Abstract

Background: The overall objective of this study was to describe the clinico-pathological spectrum of severe and non-severe vivax malaria in children and to utilize the host metabolites platform for discovery of malaria biomarkers for diagnosis of asymptomatic and low-parasitemic vivax malaria patients and its association with disease severity.

Methods: This study was carried out as a hospital based, prospective observational case-control study in two steps over a period of 12 months in the children admitted in PBM, children Hospital, Sardar Patel Medical College, Bikaner from September 2018 to August 2019.

Results: Out of 16 differentially expressed metabolites 4 were up regulated (FC>2) in NSVM children in comparison to HC. These up regulated metabolites were of fatty acid (Eleostearic acid, hydroperoxylinoleic acid) and glycosphingolipid (Hexadecasphinganine, Sphinganine) pathway. Twelve metabolites were down regulated (FC<2) in NSVM children in comparison to HC, which belonged to Amino acid pathway (arginine, glutamine, nor leucine, lysine and cystine) , Purine pathway (xanthine, arabinosylhypoxanthine, adenosine 5 monophosphate), Urea cycle pathway (citrulline) ,DHA ethyl ester(ω-3 PUFA) and vitamin B_6. Out of 14 differentially expressed metabolites 8 were up regulated (FC>2) in SVM in comparison to HC. These up regulated

metabolites were of Amino acid metabolism (valine, isoleucine, leucine), Tryptophan metabolism (kynurenine), Fatty acid metabolism (Hydroperoxylinoleic acid) and Porphyrine and Heme metabolism (urobilinogen,urobilin). Six metabolites were down regulated (FC<2) in SVM in comparison to HC, these were DHA ethyl ester (ω-3 PUFA), metabolites of carnitine shuttle (Propionylcarnitine, Acetylcarnitine), Arginine amino acid and Glycerophospholipid (Phosphocholine, L-alpha Glycerylphosphorylcholine). Hydroperoxylinoleic acid (fatty acid) was most up regulated (FC 5.4, log2FC 2.2) and Docosahexaenoic ethyl ester (ω-3 PUFA) was most down regulated (FC 0.03, log2FC -4.95) metabolites in SVM in comparison to NSVM.Glutamine amino acid was up regulated (FC 2.3, log2FC 1.2) and norleucine amino acid was down regulated (FC0.44, log2FC -1.17) in SVM in comparison to NSVM.

Conclusion: Our study identified differentially expressed metabolites and the underlying metabolic pathways linked to SVM infection. These results provide insights into the mechanisms of SVM pathogenesis, and its relation to alteration of metabolic pathways such as fatty acid ß-oxidation, purine metabolism, porphyrine and heme metabolism, tryptophan metabolism, lipid metabolism and amino acids metabolism, that might serve as therapeutic targets to alleviate SVM. Valuable information regarding the correlation of the identified metabolites with disease progression and their efficacy as disease monitoring or prognostic markers can be acquired from the more huge longitudinal investigations involving continual analysis of the children after therapeutic interventions, which could be more informative future continuation of the present study.

Keywords: Malaria, Metabolic, Pathway

Introduction

Malaria is a protozoan disease transmitted by the bite of infected female Anopheles mosquito. It is the most important human parasitic diseases.[1] The burden of malaria continues to worsen globally with a devastating impact on human health and corresponding impediment to economic improvement. Organism responsible for malaria is protozoon plasmodium. In the genus plasmodium, six species[2] have been identified of which five - namely *P. falciparum, P.vivax, P. malariae* and two sympatric species of *P. ovale* (*P.o.curtisi* and *P.o. wallikeri*), - are human malaria species that spread by the bite of an infected female *Anopheles* mosquito. Recently identified sixth species, *Plasmodium knowlesi* is primarily a parasite of macaque monkeys and may occasionally spread to human by zoonotic transmission and cause severe illness. *P. falciparum* and *P. vivax* account for over 90% of the total malaria cases worldwide. In 2018, an estimated 228 million cases of malaria occurred worldwide. Most malaria cases in 2018 were in the WHO African Region (93%), followed by the WHO South-East Asia Region (3.4%) and the WHO Eastern Mediterranean Region (2.1%). 19 countries in sub-Saharan Africa and India carried almost 85% of the global malaria burden[3]. Six countries accounted for nearly half of all malaria cases worldwide: Nigeria (25%), Democratic Republic of the Congo (12%), Uganda (5%) and Cote d'Ivoire, Mozambique and Niger (4% each).

Traditionally Plasmodium *falciparum* (Pf) is considered responsible for severe malaria and mortality due to malaria, in 2018, there were an estimated 405000 deaths from malaria globally[3]. Children aged less than 5 years are the most vulnerable group affected by malaria. In 2018, they accounted for 67% (272000) of all malaria deaths worldwide[3].

The overall objective of this study was to describe the clinico-pathological spectrum of severe and non-severe vivax malaria in children and to utilize the host metabolites platform for discovery of malaria biomarkers for diagnosis of asymptomatic and low-parasitemic vivax malaria patients and its association with disease severity.

Materials and Methods

This study was carried out as a hospital based, prospective observational case-control study in two steps over a period of 12 months in the children admitted in PBM, children Hospital, Sardar Patel Medical College, Bikaner from September 2018 to August 2019. The first step was to identify patient with *P. vivax* malaria, collect clinical data and serum samples while the second step was to subject the serum samples to metabolomics study with appropriate controls at Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India. Total 90 children with diagnosis of malaria were enrolled, 6 children having other co-morbidities along with malaria and 3 children having mixed plasmodia infection were excluded. Thus, 81 patients with plasmodium vivax mono infection were analyzed in this study.

Inclusion Criteria

1. Patients of either sex, age less than 15 years.
2. Patients with *P. vivax* mono infection confirmed with microscopic examination and/or RDT.
3. Those willing to provide written / informed consent and comply with protocol requirements.

Exclusion Criteria

1. Malaria patients with history of significant systemic diseases like autoimmune disorders, chronic liver diseases, psychiatric illness and bleeding disorder etc. as judged by history and physical examination.
2. Malaria patients having mixed infection (both *P. vivax* and *P. falciparum*)
3. Co-infection with Dengue fever, UTI, bronchopneumonia.
4. Subjects unwilling to consent for the study.

In children selected for this study, diagnosis of malaria was made with peripheral blood smear examination and rapid diagnostic test i.e. Optimal test and Falcivax test. On the day of admission, detailed history was taken regarding duration and type of fever, abdominal pain, headache, vomiting, convulsion, urine output and bleeding tendencies. Thorough clinical examination was done. Temperature, pulse, respiratory rate, blood pressure, pallor, icterus, petechiae/ecchymosis, liver and spleen size and consistency were recorded. Central nervous system examination was done in case of relevant history. All the findings were recorded on predesigned proforma. Severe or complicated malaria was defined according to WHO guidelines.

Investigations workup of all children infected with *P.vivax* monoinfection were done as Complete blood counts, Peripheral blood smear in detail, Level of parasiteamia, SGOT, SGPT, Serum bilirubin, Serum alkaline phosphatase, Serum creatinine, BUN, Random blood sugar, Bleeding time, Clotting time, Prothrombin Time (INR), Urine analysis, Fundus examination, C.S.F studies for cerebral malaria (CM), Ultrasonography (USG) Abdomen, Computerized Tomography (CT) of the head and electroencephalography (EEG) as required.

Specific test for hepatitis A, B and C in hepatic dysfunction, Glucose-6-phosphate dehydrogenase (G6PD) enzyme level for hemolysis, Chest Radiograph (CXR) and blood culture were done when necessary.

Appropriate blood tests were performed to rule out co-morbid infections like typhoid fever (widal test and blood culture) and dengue infection (differential detection of IgG and IgM antibodies).

These samples were sent to IIT Bombay, Powai, Mumbai 400076, India, for metabolomics analysis. A comparison between the severe and non-severe vivax malaria was made based on the differential expression of various metabolomics in affected children and it was compared to healthy controls (HC); these were the children of same age group, race, environment and were disease free.

Diagnosis of Malaria

As soon as malaria was suspected clinically, before administration of anti-malarial drugs, venous blood was collected and thick and thin smear were prepared.

Microscopic Diagnosis

The diagnosis of malaria was established by identification of organisms on Giemsa-stained smears of peripheral blood. Giemsa stain is superior to Wright stain or Leishman stain. Both thick and thin blood smears should be examined. The slide was considered negative when there were no parasites in the 100 high-power field. The concentration of erythrocytes on a **thick smear is 20-40** times that on a thin smear and is used to quickly scan large numbers of erythrocytes. The **thin smear** allows for positive identification of the malaria species and determination of the percentage of infected erythrocytes and is useful in following the response to therapy.

Rapid Antigen Test

OPTIMAL is a rapid malaria antigen detection test which utilizes a dipstick coated with monoclonal antibodies against the intracellular metabolic enzyme parasite lactate dehydrogenase (pLDH).

FALCIVAX is a two site sandwich immunoassay utilizing whole blood for the detection of *P. falciparum* specific histidine rich protein (Pf.HRP-2) and *P. vivax* specific pLDH.

After selection of patients /controls, 5 ml of blood sample was drawn for plasma separation. The sample was kept in ice for 30 min and then centrifuged at 2500 rpm for 15 min. After centrifugation the blood sample was divided in two parts upper yellow color liquid which is plasma and lower red color which includes cells and clotting factor. Plasma was collected in small aliquot and stored at -80^0C.

The metabolomics and targeted metabolomics analysis of the clinical samples were performed at Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India. The steps involved are described sequentially below:

1. Metabolite extraction

Serum samples were extracted in methanol in a ratio of 1: 4 (v/v). 400 µL of chilled methanol was added to 100 µL of serum, vortexed for 30 seconds and incubated at -80°C for 8 hours. The samples were then allowed to thaw on ice, followed by 15 minutes of centrifugation at 14,000X and then kept at 4°C to pellet down the precipitated protein. The resulting supernatant was evaporated in vacuum concentrator. The dried extracts were then reconstituted in 100µl of ACN : H_2O (1:1, v/v), transferred to HPLC vials and stored at -80°C prior to LC/MS analysis.

2. Instrumentation and data acquisitions

Metabolites were analyzed using an Agilent 1260 Infinity HPLC system coupled to an Agilent 6550 HRLC system consisting of dual AJS ESI probe, binary pump, on-line degasser, thermostat dual 54-well plate and autosampler (Agilent Technologies, Santa Clara, CA, USA). 1µL of the metabolite sample was injected

(using auto-sampler maintained at 4°C) onto the Agilent Zorbax SB-AQ (RP) column (100 mm X 2.1 mm, 1.8 µm). Separation was performed at 40°C with a flow-rate of 0.3 ml/min using 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in 90% acetonitrile (mobile phase B). For analysis in the positive mode, the gradient was started at 5% mobile phase, increasing linearly to 100% B in 50 minutes and then held for 60 minutes. Initial conditions were restored in 5 minutes, 10 minutes ahead of column re-equilibration. In the positive mode, a capillary voltage of 3.0 kV and a cone voltage of 175 V were applied. Data was collected in centroid mode over the mass range m/z 50–1000 with a scan rate 1.5 spectra/sec, the desolvation gas flow was 360 L/h and the source temperature was 325°C. Agilent Mass Hunter™ software was used for untargeted data acquisition.

3. Data Processing and Analysis

Analysis was performed based on the molecular feature using the mass profiler professional (MPP) version 12.6 software (Agilent Technologies, Santa Clara, CA). Data was deconvoluted into individual chemical peaks (metabolic features characterized by a unique m/z and retention time) in Agilent Mass Hunter™ Qualitative Analysis B.05.01 using molecular feature extractor (MFE), a naive or "untargeted" data-mining algorithm with an absolute height filter set to 5,000 counts; giving more details of the parameters used to define the metabolic feature. A separate, complementary "targeted" data mining approach was used that was based on a list of annotated compounds with empirical formulas. An algorithm, "Find by Mass" was used to find compounds in LC/MS data files. The list of compounds and their associated formulas were derived from the METLIN. The results of the analysis was a series of extracted ion chromatograms (EICs) that were saved as CEF files and were used for subsequent statistical analysis and data visualization in MPP software. The annotated compounds with CAS and KEGG IDs were further used for pathway search in MPP that finds pathways directly imported from wiki-pathways.

4. Data Filtering, Statistical analysis and data visualization

Separate projects were created in MPP based on the acquired data (individual patients). The data were filtered to create entity (feature) lists based on the number of entities detected in at least one condition for the HC, NSVM and SVM samples. For a given condition of disease, filtered entity lists were created based on the number of entities that passed the 70% sample replicate thresholds. Eight independent biological replicates were analyzed as one group after the ion intensity for each molecular ion was averaged across the replicates. The entities were first filtered for the frequency of detection in all biological replicates. The quality of the samples was determined by PCA analysis for all conditions. Differentially expressed feature lists between infected and healthy patients, were determined by ANOVA and identified using the METLIN PCDL. The data files were aligned and grouped in accordance to condition (infected and healthy), filtering, quality control, statistical analysis and compound identification via METLIN database matching. Unsupervised principal component analysis (PCA) was performed to visually demonstrate the variance of the metabolic phenotypes within the group on the sample files based on the infection state. ANOVA analysis resulted in lists of differentially expressed features. They were provisionally identified by matching compounds to the METLIN database.

Multivariate statistical analysis using unsupervised PCA was performed with mean centering and scaling to visually demonstrate the variance of the metabolic phenotypes from the groups based on the discriminating features from the ANOVA analysis. Statistical evaluation of the data was performed using univariate analyses, including the unpaired t-test for independent pairs of groups, and one way ANOVA for pool data. The effects of infection state on classifying the samples were compared. A cutoff value of $P<0.05$ was considered statistically significant in one-way ANOVA, using the Benjamini and Hochberg False Discovery Rate was set to 5% for multiple testing corrections .The results were summarized as a matrix table showing the comparison results for each pair of conditions as a P-value. Fold Change>2 and P-values <0.05 were the criterion to select differentially expressed metabolites. Missing values were excluded from the calculations, and the results were compared. Statistical methods used were p-value by the SPSS 2015 Software.

Provisional compound IDs were generated based on accurate mass, isotope ratios, abundances and spacing, as well as RT matching for the selected compounds. The compounds found to be significantly modulated (i) between diseased states and healthy control and (ii) among the disease states were identified in MPP by accurate mass matching, to a METLIN database of >25,000 compounds. The Agilent METLIN database currently contains over 25,000 compounds, and includes links to KEGG identifiers, CAS numbers, HMDB and LIPID MAPS identifiers. Only those metabolites whose CAS or KEGG Ids matched were selected for further analysis and pathway search. The MPP software finds pathways directly imported from wiki-pathways.

Observations

This prospective hospital based study was carried out in the children admitted in PBM, children Hospital, SPMC, Bikaner from September 2018 to August 2019. Total 81 children with *Plasmodium vivax* mono infection were analyzed in this study.

5. Metabolite identification and pathway search

Table 1: Distribution of children according to severity of malaria

Type of malaria	No.	%
Severe vivax malaria	18	22.22
Non severe vivax malaria	63	77.78
Total	81	100

In this study severe vivax malaria was found in 18 (22.22%) children.

Table 2: Age wise distribution of patients

Age (In years)	Non severe (n=63)		Severe (n=18)		Total (n=81)	
	No.	%	No.	%	No.	%
0-5	20	31.74	11	61.11	31	38.27
6-10	30	47.61	6	33.33	36	44.44
>10	13	20.63	1	5.56	14	17.28

Total	63	100	18	100	81	100
Mean age (In years) ± SD	7.30 ± 3.41		4.78 ± 2.82		6.74 ± 3.44	

The highest number of children (44.44%) were in the age group 6-10 years. In severe vivax malaria maximum number of children (61.11%) were in 0-5 year age group. The mean age was 7.30 ± 3.41 years, 4.78 ± 2.82 years for non-severe and severe vivax malaria respectively. The overall mean age of the participants included in this study is 6.74 ± 3.44 years.

Table 3: Gender wise distribution of patients

Gender	Non Severe (n=63)		Severe (n=18)		Total (n=81)		P value
	No	%	No	%	No	%	
Male	38	60.32	10	55.56	48	59.26	0.846
Female	25	39.68	8	44.44	33	40.74	
Total	63	100	18	100	81	100	

It is noticed that number of male patients was higher (59.26%). There was no significant difference in severity on the basis of gender. P value is 0.846 i.e. statistically insignificant.

Table 4: Clinical examination finding in patients with *P. vivax* malaria (N=81)

Clinical examination	No. of patients	Percentage
Fever	81	100
Pallor	42	51.85
Icterus	16	19.75
Bleeding manifestation • Epistaxis • Melena • Hematuria • Hematemesis	10 7 1 1 1	12.34
Hepatomegaly	16	19.75
Splenomegaly	16	19.75
Hepatosplenomegaly	29	35.80
Altered sensorium	1	1.23

Fever was present in all (100%) children presenting with malaria. Second most common manifestation was pallor which was present 51.85% of children. Icterus, bleeding manifestation was present in 19.75%, 12.34% respectively. On systemic examination hepatosplenomegaly, hepatomegaly, splenomegaly was present 35.80%,19.75%,19.75% respectively and altered sensorium was present in one children.

Table 5: Major manifestations of severe vivax malaria (n=18)

Manifestations	No. of patients	%
Severe Anaemia (Hb <5g/dL)	13	72.22
Renal failure (S. Creatinine >3mg/dL)	5	27.78
Jaundice (S. Bilirubin >3mg/dL)	10	55.56
Bleeding manifestations • Epistaxis • Hematemesis • Melena • Hematuria	7 4 1 1 1	38.89
Altered sensorium	1	5.56
Coma	1	5.56

We found 72.22% children had severe anaemia (<5mg/dL). Jaundice, Bleeding manifestations and Renal failure were present in 55.56%, 38.89%, 27.78% children respectively. Coma and altered sensorium was present in one child.

Table 6: Multi organ dysfunction in severe vivax malaria (n=18)

Organ dysfunction	No. of patients	%
Severe Anaemia + Jaundice	5	27.78
Jaundice + Renal failure	1	5.56
Severe Anaemia + Jaundice + Renal failure	1	5.56
Severe Anaemia + Jaundice + Renal failure + Altered sensorium	1	5.56

Hepatic dysfunction with severe Anaemia was commonest (27.78%) multi organ dysfunction.

Table 7: Distribution of cases according to fatality

Outcome	Severe		Non severe	
	No.	%	No.	%
Discharge	17	94.44	63	100
Death	1	5.56	0	0.00
Total	18	100	63	100

In our study only one death occurred, rest all recovered and were discharged from the hospital.

Metabolomics Analysis

The mechanisms that contribute to the transition from NSVM to SVM are not well understood. This present metabolomics study identified different metabolic pathways linked to malaria progression and mechanisms of SVM pathogenesis. In this study sera from children of NSVM (n=14), SVM (n=7) and HC (n=12) were analyzed via LC-MS analysis and 361 metabolites were detected. Of the 361 metabolites, 26 metabolites were differentially expressed between NSVM and SVM in comparison to HC, allowed us to discover a variety of metabolite pathways linked to

pathogenesis of SVM. These pathway belonged to fatty acid ß-oxidation, purine metabolism, porphyrine and heme metabolism, tryptophan metabolism, lipid metabolism and amino acids metabolism.

Table 8: Identified metabolites which expressed differentially in NSVM as compared to HC

S. N.	Metabolite	Pathway	Fold Change	Log2(Fc)
1	Eleostearic Acid	Fatty Acid	2.6964	1.431
2	8(R)-Hydroperoxylinoleic Acid	Fatty Acid	2.4023	1.2644
3	Hexadecasphinganine	Glycosphingolipid	2.2602	1.1764
4	Sphinganine	Glycosphingolipid	2.2202	1.1507
5	L-(+)-Arginine	Amino Acid	0.49839	-1.1642
6	L-Glutamine	Amino Acid	0.48947	-1.0307
7	Xanthine	Purine Metabolism	0.48381	-1.0475
8	Pyridoxamine 5-Phosphate	Vitamin	0.48361	-1.0481
9	L-Norleucine	Amino Acid	0.44286	-1.1751
10	L-(+)-Citrulline	Urea Cycle	0.42625	-1.2302
11	Arabinosylhypoxanthine	Purine Metabolism	0.4153	-1.2677
12	Dl-Lysine	Amino Acid	0.39375	-1.3447
13	Adenosine 5'-Monophosphate	Purine Metabolism	0.3466	-1.5287
14	L-Cystine	Amino Acid	0.33256	-1.5883
15	L-Gamma-Glutamyl-L-Lysine	Amino Acid	0.32772	-1.6095
16	Docosahexaenoic Acid Ethyl Ester	Fatty Acid	0.3103	-1.6882

Out of 16 differentially expressed metabolites 4 were up regulated (FC>2) in NSVM children in comparison to HC. These up regulated metabolites were of fatty acid (Eleostearic acid, hydroperoxylinoleic acid) and glycosphingolipid (Hexadecasphinganine, Sphinganine) pathway. Twelve metabolites were down regulated (FC<2) in NSVM children in comparison to HC, which belonged to Amino acid pathway (arginine, glutamine, nor leucine, lysine and cystine) , Purine pathway (xanthine, arabinosylhypoxanthine, adenosine 5 monophosphate) , vitamin B_6 , Urea cycle pathway (citrulline) and DHA ethyl ester(ω-3 PUFA).

Table 9: Identified metabolites which expressed differentially in SVM as compared to HC

S.N	Metabolite	Pathway	Fold Change	log2(FC)
1.	L-(+)-Valine	Amino acid	39.183	5.2922
2.	L-Isoleucine	Amino acid	14.735	3.8811
3.	L-gamma-Glutamyl-L-leucine	Amino acid	14.498	3.8578
4.	L-Kynurenine	Tryptophan metabolism	12.175	3.6058
5.	8(R)-hydroperoxylinoleic acid	Fatty acid	4.4203	2.1441

6.	Urobilinogen	Porphyrine and heme metabolism	4.1503	2.0532
7.	L-Glutamine	Amino acid	3.2894	1.7178
8.	D-Urobilin	Porphyrine and heme metabolism	3.1009	1.6327
9.	Propionylcarnitine	Fatty acid β- oxidation	0.4652	-1.1039
10.	Acetylcarnitine	Fatty acid β- oxidation	0.3182	-1.6518
11.	L-(+)-Arginine	Amino acid	0.3012	-1.7312
12.	Phosphocholine	Glycerophospholipid	0.0810	-3.5021
13.	L-alpha Glycerylphosphorylcholine	Glycerophospholipid	0.0755	-3.7269
14.	Docosahexaenoic acid ethyl ester	Fatty acid	0.0723	-3.7898

Out of 14 differentially expressed metabolites 8 were up regulated (FC>2) in SVM in comparison to HC. These up regulated metabolites were of Amino acid metabolism (valine, isoleucine, leucine), Tryptophan metabolism (kynurenine), fatty acid metabolism (Hydroperoxylinoleic acid) and Porphyrine and Heme metabolism (urobilinogen,urobilin). Six metabolites were down regulated (FC<2) in SVM in comparison to HC, these were DHA ethyl ester (ω-3 PUFA), metabolites of carnitine shuttle (Propionylcarnitine, Acetylcarnitine), Arginine amino acid and Glycerophospholipid (Phosphocholine, L-alpha Glycerylphosphorylcholine).

Table 10: Identified metabolites which expressed differentially in SVM as compared to NSVM

S.N.	Metabolite	Pathway	Fold Change	log2(FC)
1.	8(R)-hydroperoxylinoleic acid	Fatty acid	5.4203	2.2383
2.	L-Glutamine	Amino acid	2.2894	1.1949
3.	L-Norleucine	Amino acid	0.44286	-1.1751
4.	Docosahexaenoic acid ethyl ester	Fatty acid	0.0323	-4.9523

Hydroperoxylinoleic acid (fatty acid) was most up regulated (FC 5.4, log2FC 2.2) and Docosahexaenoic ethyl ester (ω-3 PUFA) was most down regulated (FC 0.03, log2FC -4.95) metabolite in SVM in comparison to NSVM. Glutamine amino acid was up regulated (FC 2.3, log2FC 1.2) and norleucine amino acid was down regulated (FC0.44, log2FC -1.17) in SVM in comparison to NSVM.

Discussion

India contributes a significant burden of *P. vivax* malaria in the world. The infection due to *P. vivax* poses a greater challenge to roll back malaria programme as transmission of this parasite is hard to control, largely because of dormant hypnozoite stages. Severe malaria is classically associated with *P. falciparum* monoinfection, however recent studies have broken the myth that vivax malaria is benign in nature and is now recognized as a cause of severe and fatal disease[4].

This prospective study was carried out in department of Paediatrics, S.P. Medical College, Bikaner to study clinico-laboratory profile with clinical outcome of

admitted children suffering from *P. vivax* malaria and to evaluate the correlation of identified metabolites with disease severity. We enrolled 81 children of *P. vivax* monoinfection in our study. On the basis of the WHO criteria for severe malaria, 18 children (22.22%) were identified to have severe vivax malaria. Similar higher proportion of complicated *P. vivax* malaria (50%) has been reported by Mittal et al[4]. Dougles et al[5] conducted a similar study in Papua, Indonesia, reported severe malaria in 24% children with *P. vivax* infection and 23% children with *P. falciparum* infection. So severe malaria caused by vivax is an emerging entity and challenges the perception of *P. vivax* malaria as a benign disease.

In our study percentage of severe anaemia (<5 gm%) was 16.04% and in children with severe P. vivax malaria, severe anaemia was 72.22%.

Mean haemoglobin was 8.60 ± 1.91gm/dL. In severe vivax malaria mean Hb was 5.51±1.86 gm/dL. A lower prevalence of anaemia was found in Mittal et al[47] study (33.1%) and Kwenti et al[6] study (29.3%). Anaemia is associated with haemolysis, but removal of infected erythrocytes by the spleen and impairment of erythropoiesis are likely to play a greater role than hemolysis in the pathogenesis of severe malarial anaemia[7].

Renal dysfunction/acute kidney injury was found in 6.17% (5/81). Ultrasonography of abdomen showed normal kidneys without any parenchymal lesion; while in Mittal et al[4] study it was 3.7% which was lower to that found in our study. Singh et al[8]reported renal dysfunction in 17.6% children which was higher than that found in our study.

Shock or circulatory collapse can be of two types: compensated with no hypotension or decompensated with systolic blood pressure <70 mm of Hg. It was found in 1.23% (1/81) children ; with inotrope requirement in one (1.23%). Shock was found in 8.5% in Mittal et al[4] study and 11.7% in Kwenti et al[6] study; both being higher in prevalence than found in our study.

Multi organ dysfunction (involvement of two or more than two organs) was present in 9.81% (8/81) children whereas it was 2.8% in Mittal et al[4] study; a lower prevalence than our study. Most common combination was anaemia and hepatic dysfunction.

Mortality was 1.23% (1/81) of a 5 years old male child. The child who died because of severe *P. vivax* malaria had MODS with cerebral malaria; had liver dysfunction and altered consciousness. Mittal et al[4] reported mortality of 4.7% with 60% deaths from *P. vivax* and remaining with Pf; higher than that found in our study. It was 5.8% in Kwenti et al[6] study; all deaths were due to PF malaria infection and majority being below 5 years of age.

In this study we found many amino acid derangements in vivax malaria. In this study arginine, glutamine, nor leucine, lysine and cystine were down regulated (FC<2) in NSVM but valine, isoleucine, leucine, tryptophan metabolite (Kynurenine) and glutamine were up regulated (FC>2) in SVM in comparison to HC. In this study, glutamine was found to be down regulated (FC 0.48, log2FC-1.03) in NSVM in comparison to HC. Glutamine is a conditionally essential amino acid that is required by rapidly dividing cells such as leucocytes and enterocytes, particularly in catabolic states[9,10]. Glutamine has a wide spectrum of other biological activities, such as renal ammonia genesis[11], glutathione synthesis[12] and carbohydrate metabolism[13,14]. Low plasma glutamine levels have been reported in a number of conditions, including burns[15], critically ill patients[16], trauma, sepsis[17], and

premature infants[18,19]. Low plasma glutamine levels are a result of higher glutamine utilization in catabolic tissues, which is not compensated by for increased proteolysis[20]. But glutamine was up regulated in SVM in comparison to HC (FC 3.28, log2FC 1.71) and NSVM (FC 2.28, log2FC 1.19), There are several possible explanations for this finding: there may be increased glutamine release into plasma in severe malaria; plasma clearance of glutamine may be decreased; the volume of distribution of glutamine may have decreased; or a combination of mechanisms may operate.

Amino acid arginine was down regulated in` NSVM (FC 0.49, log2FC -1.16) and SVM (FC 0.30, log2FC -1.73). Arginine plays an important role in cell division, removing ammonia from body, immune function and it is precursor for the synthesis of nitric oxide. L-arginine deficiency was attributed to increased metabolism of L-arginine following intravascular nitric oxide depletion and by arginases from host[21] or parasite[22,23]. It has recently been proposed that L-arginine deficiency might arise from low bioavailability of its precursors[24]. We measured two L-arginine precursors, ornithine and citrulline, and observed both were significantly depleted in uncomplicated malaria. Our findings support the hypothesis that low-bioavailability of precursors contributes to L-arginine deficiency.

Other significant changes occur in pathways such as the carnitine shuttle and lipid metabolism. The palmitoylcarnitine (FC 0.46, log2FC-1.10), acetylcarnitine (FC 0.32, log2FC-1.65), were down regulated with SVM**.** These metabolites are involved in the carnitine shuttle pathway. The transfer of long-chain fatty acids across the inner mitochondrial membrane for β-oxidation is mediated by the carnitine shuttle pathway, whereas decreased abundance of related metabolites identified here might reflect increased uptake of host fatty acids by the parasite[25]. Consistent with this hypothesis, we observed that the level of metabolites such as glycerophosphocholine (FC 0.07, log2FC -3.72) and phosphocholine (FC 0.08, log2FC -3.72) were also down regulated with SVM . Liver stage parasites require phosphatidylcholine for growth and this may also contribute to decrease blood levels. In contrast, we identified a significant increase in oleic acid (hydroperoxylinoleic acid) (FC 5.42, log2FC 2.24) in SVM **,** a fatty acid that is required for the intra-erythrocytic proliferation and essential for the growth of *P. vivax*[26]. Indeed, malarial parasites scavenge, modify, and incorporate fatty acids and phospholipids from the host[27], whereas elevated *P. vivax* parasitemia correlates with reduced levels of low and high-density lipoprotein in the serum of patients[28]. Lipids are essential nutrients for parasite's proliferation, and for the conversion of heme into hemozoin[29], which is a detoxification strategy used by the parasite to survive inside the RBC while digesting hemoglobin. In this study stearic acid (eleostearic acid) was up regulated (FC>2) in NSVM and Docosahexanoic acid ethyl ester, a ω-3 PUFA was down regulated in both NSVM (FC 0.31, log2FC -1.38),and SVM (FC 0.07, log2FC -3.78). It is assumed that during the blood stage of malarial infection the Plasmodium vivax reproduces in a safe haven, the food vacuole, totally isolated from the hostile outside world of host defenses[30]. Nevertheless the parasite must import a multitude of substances and export others. To this effect it grossly alters the structure and composition of the host erythrocyte with change in phospholipid and fatty acid compositions as large increases in palmitic acid, stearic acid, oleic acid and major decreases in arachidonic acid and docosahexaenoic (DHA)[31].

In Porphyrine and heme metabolism urobilinogen (FC 4.15, log2FC2.05) and urobilin (FC3.10, log2FC1.63) were up regulated in SVM**.** Some of the top predicted metabolites such as biliverdin, bilirubin, bilirubin-glucoronoside, urobilinogen, urobilin and bilirubin beta-diglucuronide are involved in porphyrin and heme metabolism. These metabolites exhibited higher abundance in the plasma of patients with SVM. Malaria has been characterized by hemolysis of both infected and uninfected erythrocytes[32] leading to the release of cell-free hemoglobin and further heme prosthetic groups[33]. Noteworthy, patients with P. vivax malaria exhibit elevated levels of cell-free hemoglobin in plasma[34] and up-regulate levels of heme oxygenase-1 (HO-1)[35], which breaks down heme into biliverdin, carbon monoxide and iron. Levels of HO-1 and several other immunomodulatory and inflammatory mediators also correlate with *P. vivax* severe disease.

Conclusion

Our study identified differentially expressed metabolites and the underlying metabolic pathways linked to SVM infection. These results provide insights into the mechanisms of SVM pathogenesis, and its relation to alteration of metabolic pathways such as fatty acid ß-oxidation, purine metabolism, porphyrine and heme metabolism, tryptophan metabolism, lipid metabolism and amino acids metabolism, that might serve as therapeutic targets to alleviate SVM. Valuable information regarding the correlation of the identified metabolites with disease progression and their efficacy as disease monitoring or prognostic markers can be acquired from the more huge longitudinal investigations involving continual analysis of the children after therapeutic interventions, which could be more informative future continuation of the present study.

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