Peer Review File

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Review Comments:

Comment 1: The manuscript lacks detailed information on the severity of sepsis of the patients in this study group. There is also no information on the timing of the blood sampling by means of how long the patients had been symptomatic until the blood was drawn.

Reply1(1): Thanks for your comments. Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. The predominant score in current use is the Sequential Organ Failure Assessment (SOFA) (originally the Sepsis-related Organ Failure Assessment. A higher SOFA score is associated with an increased probability of mortality. Organ dysfunction can be identified as an acute change in total SOFA score ≥2 points consequent to the infection. In out-of-hospital, emergency department, or general hospital ward settings, adult patients with suspected infection can be rapidly identified as being more likely to have poor outcomes typical of sepsis if they have at least 2 of the following clinical criteria that together constitute a new bedside clinical score termed quick SOFA (qSOFA): respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure of 100 mm Hg or less. This *qSOFA* provides simple bedside criteria to identify adult patients with suspected infection who are likely to have poor outcomes. (JAMA. 2016 Feb 23;315(8):801-10.).

Scoring systems are used for mortality and morbidity rating in intensive care conditions, prognosis prediction, standardization of scientific data and the monitoring of clinical quality, such as Acute Physiology and Chronic Health Evaluation (APACHE) II and III scoring systems. (Crit Care Med.1985 Oct;13(10):818-829. ,Chest.1991Dec;100(6):1619-1636., Ther Apher Dial.2019 Oct;23(5):418-424.). In the intensive care unit, we often use the APACHE II and III scoring system to assess the severity of the patient's disease to help evaluate interventions.

We have modified and added in the revised manuscript as following:

"…For each patient in this study, we used the Sequential Organ Failure Assessment (SOFA) score, quick SOFA (qSOFA) (Singer, M, JAMA.2016 Feb 23 ;315(8) :801-10), and the Acute Physiology and Chronic Health Evaluation (APACHE) II and III scores (Knaus WA, Crit. Care Med. 1985 Oct;13(10))(Knaus WA, Chest 1991

Dec;100(6))(Karagoz S, Ther Apher Dial 2019 Oct;23(5)] to assess sepsis severity (Tab. 1)."(Page 5, Line 4-7, red marker)

Reply 1(2): Patients with sepsis were diagnosed according to the definition of sepsis-3. Patients diagnosed with sepsis need to complete the blood lactic acid level test, obtain blood culture samples before the application of antibiotics, use broad-spectrum antibiotics, liquid resuscitation and other measures within 1 hour of hospitalization. Patients with sepsis can be identified within 1 hour of hospitalization. Patients diagnosed with sepsis were included in this study and blood samples were drawn. We did not describe it clearly. I will add a description in the manuscript and add references.
Changes in the text: We have modified our text.
And we added as following:
"…The inclusion criterion was a patient diagnosed with sepsis according to the Sepsis-3 definition (Singer Mervyn, JAMA, 2016, 315(8): 801-10.) within 1 hour of hospitalization. The exclusion criteria were the following: (i) Patient's with incomplete or inaccurate data. Since this study is a paired statistical analysis, complete data of each experimental object collected were required. If the data of the experimental object were incomplete, the collected samples could not be used in the range. (ii) Patients diagnosed with chronic metabolic diseases. According to Sepsis-3, the presence of organ dysfunction is also an important condition for the diagnosis of sepsis, and was a key diagnostic criterion in our study. For patients diagnosed with sepsis, plasma samples were collected within 1 hour of hospitalization, during the period before antibiotic treatment."(Page 4, line 17-24 and Page 5, line 1-2, red marker)

<u>Comment 2:</u> When did the organ dysfunctions occur? Before the blood was drawn or during the whole of the septic episode?

Reply 2: Firstly, patients can were diagnosed as sepsis according to the definition of sepsis-3. The presence of organ dysfunction is an important condition for the diagnosis of sepsis in "sepsis-3". We diagnosed sepsis when the patient was hospitalized, and the organ dysfunction appeared at that time. The time point of our study is instantaneous, not a continuous. This choice was based on the aim of this research which is looking for evidence for the judgment of sepsis, not the evidence for dynamic assessment of sepsis.

We have modified and added in the revised manuscript as following:

"…According to Sepsis-3, the presence of organ dysfunction is also an important condition for the diagnosis of sepsis, and was a key diagnostic criterion in our study. "(Page 4, line 23-24, red marker)

Comment 3: The manuscript deals with a lot of statistical methods. This methodology needs to be explained more in detail and the approach should be referenced to make it clearer to the reader.

Reply 3: Regarding the statistical methods in the manuscript, we did not describe it clearly. We redescribe the statistical method in the new manuscript and annotate references.

We have modified and added in the revised manuscript as following:

"…Automated Mass Spectral Deconvolution and Identification System (AMDIS) software was employed to deconvolute GC–MS chromatograms and identify metabolites using MCF mass spectra library (Smart Kathleen F, Nat Protoc, 2010, 5(10): 1709-29). The identifications were based on the both MS spectrum of the derivatized metabolite and its respective chromatographic retention time. The relative abundance of identified metabolites was determined by ChemStation (Agilent) by using the GC base-peak value of a selected reference ion. These values were normalized using the biomass content in each sample and internal standard abundance. Student's t-test was used to determine whether the relative abundance of each identified metabolite was significantly different between sepsis samples and controls. The partial least squares discriminant analysis (PLS-DA) and receiver operating characteristic (ROC) curves were drawn using Microsoft Excel (Microsoft Corporation, USA) and inserted into Multibase (Digital Dynamics, Japan). Our Pathway Activity Profiling (PAPi) algorithm [20] was used to predict and compare the relative activity of different metabolic pathways in sepsis. This program is linked to the Kyoto Encyclopedia of Genes and Genomics (KEGG) online database (http://www.kegg.com) and uses the number of metabolites identified from each pathway and their relative abundances to predict which metabolic pathway is likely to be active in sepsis. The entire data mining, data normalization, and pathway activity predictions were automated by our in-house R software package as described in Smart et al. (Aggio Raphael B M, Bioinformatics, 2010, 26(23): 2969-76). Graphical representations of the results were produced by ggplot2 R packages. In addition, correlation analysis of metabolites in various

subgroups and clinical indicators was performed. Statistical analysis was performed using GraphPad Prism 6.0."(Page 6, line 23-25, and Page 7, line 1-20, red marker)

Comment 4: Where there metabolites that did only occur in sepsis patients? Which were they?

Reply 4: Among these different metabolites, we found that 26 metabolites were fatty acids, which included branched fatty acids (3), saturated fatty acids (10), and unsaturated fatty acids (13) that were found in sepsis plasma samples but not in the controls. (see Page 8, line 8-11).

The 26 fatty acids were as follows: (see Fig. 2D)

branched fatty acids (3):

3-methyl-2-oxopentanoic acid; 4-methyl-2-oxopentanoic acid; tetradecanoic,12-methyl,methyl ester.

saturated fatty acids (10):

10,13-dimethyltetradecanoic acid; Arachidic acid; DPA; Hexanoic acid; Margaric acid; Nonadecanoic acid; Palmitic acid; Pentadecanoic acid; Propanedioic acid, methyl, ethyl ester; Stearic acid

unsaturated fatty acids (13):

11,14-Eicosadienoic; 11,14,17-Eicosatrienoic acid; 2-Methyloctadecanoic acid; 3-Hydroxyoctanoic acid; Adrenic acid; Arachidonic acid; bishomo-gamma-Linolenic acid; Conjugated linoleic acid; DHA; EPA; Linoleic acid; Myristoleic acid; Palmitoleic acid.

We have added it in the manuscript as following:

"…Of these, 26 were fatty acids, including 3 branched fatty acids, 10 saturated fatty acids, and 13 unsaturated fatty acids, which were found in sepsis plasma samples but not in the controls (Fig. 2D). The 3 branched fatty acids included 3-methyl-2-oxopentanoic acid; 4-methyl-2-oxopentanoic acid; and tetradecanoic acid, 12-methyl, methyl ester. The (B) 10 saturated fatty acids included 10,13-dimethyltetradecanoic acid; arachidic acid; docosapentaenoic acid; hexanoic acid; margaric acid; nonadecanoic acid; palmitic acid; pentadecanoic acid; propanedioic acid, methyl, ethyl ester; and stearic acid. The 13 unsaturated fatty acids included 11,14-eicosadienoic acid; 11,14,17-eicosatrienoic acid; 2-methyloctadecanoic acid; 3-hydroxyoctanoic acid; adrenic acid; arachidonic acid; bishomo-gamma-linolenic acid; conjugated linoleic acid; docosahexaenoic acid (DHA); eicosapentaenoic acid (EPA); linoleic acid;

myristoleic acid; and palmitoleic acid (Fig. 2D)." (Page 8, line 8-20 and Figure legend (Fig. 2D)).

Comment 5: **(5) As you deal with substance levels: Did you calibrate the GC-MS on the substances or did you calculate the plain signal intensities you measured?**

Reply 5: We have added in the revised manuscript as following:

"…The relative abundance of identified metabolites was determined by ChemStation (Agilent) by using the GC base-peak value of a selected reference ion. These values were normalized using the biomass content in each sample and internal standard abundance." (Page 7, line 2-5, red marker)

Comment 6: **(6) I would suggest to move the subgroup analyses of organ dysfunctions the supplement of the paper on focus on the sepsis vs. non-sepsis part.**

Reply 6: Thanks for your comments. We conduct a subgroup analysis of organ dysfunction caused by sepsis in order to provide important clinical hints. However, this part occupies too much space in the article, especially for small sample matching studies, and it will also cause some clinical troubles. Therefore, we focus on the differential products of sepsis subgroups mainly and draw a complete table. For the subgroup statistical analysis AUC, correlation analysis and other data, they are placed in the corresponding attachments. In the discussion section, we will discuss the results of special and important subgroups appropriately.

Changes in the text: We have modified with advised in manuscript as following:

"…3.2 Subgroups associated with sepsis-related organ dysfunction (Table 2)

A significant difference in phenylalanine levels was observed between the AKI and non-AKI groups. The present study identified seven metabolites that differed between these groups (Supplemental Fig. 1, Supplemental Table 1). Furthermore, notable differences in the levels of three metabolites were observed between the ARDS and the non-ARDS groups, including 3-hydroxydecanoic acid, β-methylamino-l-alanine (BMAA), and 1H-imidazo[4,5-b] pyridine-2-carboxaldehyde (Supplemental Fig. 2, Supplemental Table 2). Moreover, substantial differences in the levels of glutamine were observed between the SIMD and non-SIMD groups. In addition, seven metabolites also showed significant differences between these two subgroups. Only glutamine showed a positive correlation with the clinical indicator CK-MB (Supplemental Fig. 3, Supplemental Table 3). A marked difference in phenylalanine

levels was observed between the AHI and non-AHI groups. 3-methyl-2-oxopentanoic acid and 2-coumaranone were negatively correlated with total bilirubin (TBIL), while phenylalanine was positively correlated with TBIL (Supplemental Fig. 4, Supplemental Table 4)." (Page 10, line 14-25 and Page 11, line 1-3. Table 2)

Comment 7: **(7) The blood samples in the study are not more than a snap shot of one patient. This limitation should be pointed out very clearly. It would be interesting to investigate the trends of potentially sepsis-related metabolites.**

Reply 7: Thanks for your comments, we have added this limitation in discussion as following:

"…Some limitations of the present study should be noted. Firstly, we utilized a small study population. However, we matched the included sepsis patients with the controls, which could eliminate the interference caused by the small sample size to some extent. Furthermore, this paired analysis method is recognized in metabolomics research (Talanta.2019 Sep 1;202:572-579). Our research object was a sample group, and through the pairing of gender, age, and body mass index, a standardized paired sample could be established. However, this pairing method also has certain limitations, as the population could have had genetic polymorphisms, host differences, and other issues."(Page 16, line 11-18, red marker)

Comment 8: **The figures and tables are to detailed and do not focus on the main results. Those should be presented in a well-arranged and more focused manner.**

I also suggest to revise the following minor points:

(1) Abstract: line 12 should read: "Despite advances"

Reply (1): Thanks for your comments. We have modified our text as advised. (Page 1, line 12, red marker)

(2) Abstract: the methods section should focus on the GC-MS analytical part and the statistical analyses rather than the subgroup analyses

Reply (2): Thanks for your comments. We have modified the method part of the abstract as following: "…Plasma samples from 31 patients with sepsis and 23 healthy individuals of comparable age, gender, and body mass index (BMI) were collected. Plasma metabolites were detected through gas chromatography–mass spectrometry (GC–MS), and relevant metabolic pathways were predicted using the Kyoto

Encyclopedia of Genes and Genomics (KEGG) pathway database. Student's t-test was employed for statistical analysis. In addition, to explore sepsis organ dysfunction, plasma samples of sepsis patients were further analyzed by metabolomics subgroup analysis according to organ dysfunction." (Page 1, line 14-21)

(3) Abstract: Page 2, line 2: Use "clinical marker" instead of "clinical evidence"
Reply (3): Thanks for your comments. We have modified our text as advised. (Page 2, line 11)

(4) Methods: A registration number should be noted for the Ethics statement
Reply (4): We have indicated the registration number in the ethics approval.
Changes in the text: We have modified our text as advised. (Page 4, line 7-10)

(5) Methods: Inclusion and exclusion criteria should be stated in detail. Was there a cut off for the definition of sepsis?
Reply (5): I have added inclusion and exclusion criteria in the manuscript. There was a cut off for the definition of sepsis.
We have added inclusion and exclusion criteria in the revised manuscript as following: "…The inclusion criterion was a patient diagnosed with sepsis according to the Sepsis-3 definition (Singer Mervyn, JAMA, 2016, 315(8): 801-10.) within 1 hour of hospitalization. The exclusion criteria were the following: (i) Patient's with incomplete or inaccurate data. Since this study is a paired statistical analysis, complete data of each experimental object collected were required. If the data of the experimental object were incomplete, the collected samples could not be used in the range. (ii) Patients diagnosed with chronic metabolic diseases." (Page 4, line 17-22, red marker)

(6) Methods: Page 5: The abbreviation should be explained once before they are used in the main manuscript.
Reply (6): We have explained the use of abbreviations in manuscript.

(7) Methods: Page 5: The definitions for each organ dysfunction should be stated in detail. Information on how many patients were included in which subgroup analysis is rather results than methods.

Reply (7): Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. The predominant score in current use is the Sequential Organ Failure Assessment (SOFA) (originally the Sepsis-related Organ Failure Assessment. SOFA score ≥ 2 points indicates organ dysfunction. Regarding organ dysfunction, there is a detailed introduction in the new version of the Sepsis 3.0 International Guide. (*The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016 Feb 23;315(8):801-10*). The number of patients in the sepsis subgroup was shown in Table 1.

We have modified and added in the revised manuscript as following:

"…The acute kidney injury (AKI) (n = 16) and non-AKI (n = 15) subgroups were separated from the sepsis group based on criterion from the Kidney Disease Improving Global Outcomes (KDIGO) consensus conference, which defined markers of kidney damage or glomerular filtration rate (GFR) as <60 ml/min per 1.73 m2 for <3 months (Levey Andrew S, Kidney Int., 2020, 97(6): 1117-29). The acute respiratory distress syndrome (ARDS) (n = 22) and non-ARDS (n = 9) subgroups were separated from the sepsis cohort based on the oxygenation index according to the Berlin ARDS definition: partial pressure of oxygen (PaO2) / fraction of inspired oxygen (FiO2) <300 mmHg (Force ADT, Jama 2012, 307(23):2526-2533). As there is no definitively agreed upon definition or criteria for sepsis-induced myocardial dysfunction (SIMD), diagnosis can be difficult. However, some studies have shown that troponin (cTn) and brain natriuretic peptide (BNP) have diagnostic and prognostic value in SIMD (L'Heureux Michael, Curr Cardiol Rep, 2020, 22(5): 35). We therefore separated SIMD (n = 17) and non-SIMD (n = 14) subgroups from the sepsis cohort based on the levels of myoglobin (MB), creatine kinase–myocardial band (CK-MB) isoenzyme, n-terminal pro-BNP (NT-proBNP), and troponin-I (cTnI) (Jain A, Journal of tropical pediatrics 2018). Acute hepatic ischemia (AHI) associated with sepsis was defined by a serum bilirubin level greater than 2 mg/dL, with elevation of the serum glutamic oxaloacetic transaminase and lactic dehydrogenase levels about twice that of normal values (Fry D E, Arch Surg, 1980, 115(2): 136-40)(Levy MM, Critical care medicine 2003, 31(4):1250-1256). Therefore, the AHI (n = 19) and non-AHI (n = 12) subgroups were separated from the sepsis cohort based on serum bilirubin and amino-transferase levels. All plasma samples in each subgroup were matched to healthy volunteers in terms of age, BMI, and gender." (Page 6 line 2-21)

(8) The language of the manuscript should be revised before re-submission.

Reply (8): We have used AME Editing Service for language editing. The language editing certificate is attached.