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RESEARCH ARTICLE

A Preliminary Nuclear Magnetic Resonance Metabolomics Study Identifies Metabolites that Could Serve as Diagnostic Markers of Major Depressive Disorder

Ibrahim Mohammed Badamasi[1,#], Maulidiani Maulidiani[2,$], Munn Sann Lye[3], Normala Ibrahim[4], Khozirah Shaari[2] and Johnson Stanslas[1,*]

[1]*Pharmacotherapeutics Unit, Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia;* [2]*Laboratory of Natural Products Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia;* [3]*Department of Community Health, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia;* [4]*Department of Psychiatry, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia;* [#]*Present address of first author: Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, Bayero University Kano;* [$]*Present address of this author: Faculty of Science and Marine Environment, Universiti Malaysia Terengganu*

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***Abstract*: *Background*:** The evaluation of metabolites that are directly involved in the physiological process, few steps short of phenotypical manifestation, remains vital for unravelling the biological moieties involved in the development of the (MDD) and in predicting its treatment outcome.

***Methodology*:** Eight (8) urine and serum samples each obtained from consenting healthy controls (HC), twenty-five (25) urine and serum samples each from first episode treatment naïve MDD (TNMDD) patients, and twenty (22) urine and serum samples each s from treatment naïve MDD patients 2 weeks after SSRI treatment (TWMDD) were analysed for metabolites using proton nuclear magnetic resonance (1HNMR) spectroscopy. The evaluation of patients' samples was carried out using Partial Least Squares Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLSDA) models.

***Results*:** In the serum, decreased levels of lactate, glucose, glutamine, creatinine, acetate, valine, alanine, and fatty acid and an increased level of acetone and choline in TNMDD or TWMDD irrespective of whether an OPLSDA or PLSDA evaluation was used were identified. A test for statistical validations of these models was successful.

***Conclusion*:** Only some changes in serum metabolite levels between HC and TNMDD identified in this study have potential values in the diagnosis of MDD. These changes included decreased levels of lactate, glutamine, creatinine, valine, alanine, and fatty acid, as well as an increased level of acetone and choline in TNMDD. The diagnostic value of these changes in metabolites was maintained in samples from TWMDD patients, thus reaffirming the diagnostic nature of these metabolites for MDD.

Keywords: [1]H NMR, metabolomics, urine, serum diagnosis, prognosis, MDD.

1. INTRODUCTION

Multifactorial disorders like major depression disorder (MDD), a mood disorder, require a multidisciplinary approach to unravel their aetiology, and novel drug targets with the potential mechanism of action, efficacy, and safety profile of drugs [1, 2]. The interaction between serotonergic and adrenergic pathways in the brain and the gut had been implicated in mood changes. These interactions involve the enterochromaffin cells (ECs), the enteric nervous system (ENS), autonomic nervous system (ANS), hypothalamus-pituitary-adrenal (HPA) axis, and the central nervous system (CNS) [3].

Metabolic signatures representing the totality of genomic, symbiotic, parasitic, environmental, and co-metabolic interactions within the biological systems may be described by evaluating bio fluids, such as urine and serum [4, 5]. Potential biomarkers discovery can be elucidated using advanced analytical technologies and protocols of metabolomics [6, 7]. In addition, novel metabolites, potential targets, and a network of reactome pathways attributed with on-target and off-target activities of medications may also be identified following a successful metabolomic assessment - a key feature of pharmacometabolomics [5, 8].

*Address correspondence to this author at the Pharmacotherapeutics Unit, Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia; E-mails: rcxjs@upm.edu.my, jstanslas@yahoo.co.uk

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Multivariate analysis, including principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA), and orthogonal-projection to latent structure-discriminate analysis (O-PLS-DA), are central to metabolomics evaluations. Principal component analysis (PCA), an unsupervised assessment, was commonly used to highlight the pattern of clustering for samples when there were no secondary classification features available for the analysis. Partial Least Square Discriminant Analysis (PLS-DA) and orthogonal projections to latent structures-discriminant-analysis (OPLS-DA) are supervised methods that maximize the variations between the different study groups based on the class information, thus enumerating the metabolites involved in the separation of the samples based on class identifiers. Therefore, multivariate analysis is vital for comparison, visualizations, and discriminations of outcome-based studies entirely on differences in underlying metabolites [9]. In the current study, evaluating the metabolites in urine and serum samples of healthy controls (HC), treatment-naïve first-episode MDD (TNMDD) and treatment-naïve first-episode MDD patients treated with SSRI for 2 weeks (TWMDD), are hypothesized to harbor different metabolites that may predict each phenotype reliably.

2. MATERIALS AND METHODS

2.1. Chemical Reagents and Equipment

Reagents used in this study include 3-trimethylsilylproipionic-2,2,3,3-d4 acidic sodium salt (TSP), molecular grade sodium chloride, sodium deuterium oxide (NaOD), and phosphate buffer solution. Equipment used include a -80 °C freezer, 5 mm NMR tubes, the centrifuge machine, Varian unity INOVA 500 MHz spectrometers (Varian Inc, CA), sterile plain bottles, PH meters, the SIMCA software, the Kinomics software, pipettes, conical flasks, and ice packs.

2.2. Study Design

2.2.1. Subject Recruitment

This study is a pilot study to identify validated metabolites identifiable using nuclear magnetic resonance (NMR) for the determination of MDD as well as its treatment outcomes among newly diagnosed patients within the first 2 weeks of treatment. Healthy controls were recruited among persons visiting the ear, nose, and throat outpatient Department of Hospital Kuala Lumpur and the Surgery Department of Hospital Kajang. All treatment Naïve patients recruited in this study had been completely oblivious of MDD diagnosis and were not on any MDD treatment until they came to the hospital with complaints that suited the diagnosis for MDD identified by a trained physician, thus they were never on any MDD treatment regimen before recruitment.

Ethical approval for the study was awarded by the Medical Research and Ethics Committee (MREC) of Malaysia (NMRR-14-688-19696). Cases were recruited from psychiatric clinics in the Hospital Kuala Lumpur, Hospital Kajang, Hospital Serdang, and Hospital Putrajaya. Senior Psychiatrists and Medical officers with long-standing experience in the diagnosis and management of psychiatric cases were responsible for establishing the MDD diagnosis in line with the diagnostic criteria of the fourth Diagnostic and Statistical Manual of Mental Disorders (DSMIV) before patients were recruited [10]. Recruitment of patients following the diagnosis of MDD was done by a team of well-motivated and trained research assistants and a medically qualified postgraduate student (IBM).

The recruitment process started with obtaining the informed consent of potential participants. Taking informed consent entailed that patients were enlightened on the goals and objectives of the study. Detailed explanations were readily offered for any questions the volunteering study participants asked. The roles the study participants were expected to play in fulfilling the objectives of the study were described in great detail to each one of them. Responding to questionnaires before and after the commencement of SSRI antidepressant medications, fasting blood sampling before and at 2 weeks after the commencement of medications, strict adherence to the medications, and reporting of adverse drug reactions formed part of the responsibilities of the study participants that were emphasized at recruitment and the subsequent visits. The Montgomery Asberg depression rating scale-self (MADRS-S) questionnaire was administered to the patients at the point of recruitment before treatment onset (baseline) and by the second visit (2 weeks after treatment onset) in order to ascertain their responses to treatment. The diary enumerating a number of potential adverse effects commonly associated with SSRI treatment of MDD, which is similar to the patient-rated inventory of side effects (PRISE), was given to each study participant at baseline. The patients were instructed to carefully highlight any of the adverse effects that they experience in the diary during the next 2 weeks of treatment, and it was retrieved during the second visit (2 weeks after treatment onset) to extract the adverse effect data into the PRISE questionnaire. It is pertinent to note that the PRISE questionnaire was collaboratively filled up using a semi-assisted approach relying on patients' information from the diary as well as their hospital records.

2.2.2. Inclusion Criteria for Cases

Malaysians, 18-65 years, newly diagnosed with MDD by a trained psychiatrist or MDD patients on SSRI treatment for 2 weeks and a short course of any sedative-hypnotics, were included in this study.

2.2.3. Inclusion Criteria for Control

Participants should be free of any mental health disorder both in the present and the past. Stable medical conditions, such as diabetes mellitus and hypertension are not contraindications for recruitment.

2.2.4. Exclusion Criteria for Cases

Non-Malaysians, those with cognitive impairments, less than 18 or older than 65 years, diagnosed with axis 1 psychiatric disorders other than MDD or receiving non-SSRI medications (except sedative-hypnotics) were barred from participating in the study.

2.2.5. Exclusion Criteria for Controls

Patients diagnosed with mental illnesses in the past, currently diagnosed, and are undergoing management for mental health disorders (or not), as well as patients with unstable medical conditions, were excluded from the study.

2.2.6. Blood and Urine Sampling

Trained and qualified hospital staff volunteered in carrying out blood sampling for the consenting patients. Fasting blood and urine samples were collected after patients' diagnosis for MDD was established at the first visit. Healthy vol-

unteers who consented to participate in this study as controls were sampled in the recruiting clinics by staff appointed by the hospital to carry out the task. Routinely, patients' hospital visits after the first one was scheduled for the next 2 weeks by the managing physician to ascertain the clinical efficacy and tolerance of the prescribed medications. A repeat sampling for fasting blood and urine samples was conducted during second hospital visit. Three milliliters (3 mls) of blood samples were collected in sterile plain bottles, while a variable quantity of mid-stream urine was collected in sterile urine universal tubes. All samples were collected during the morning clinic session (between 8.00 am to 12.00 pm).

2.2.7. Transportation and Storage of Samples

Samples were transported in ice-containing packs to the Pharmacotherapeutics lab of UPM for further processing and storage. Storage in a -80°C freezer in the laboratory was carried out after centrifuging the samples at 1500 g for 10 minutes. Experiments were carried out at the Institute Biosains, Department of Natural Products of the University Putra Malaysia.

2.2.8. Questionnaires

English and Malay versions of the MADRS-S questionnaires were obtained and utilized in this study because both languages are widely spoken in Malaysia. English and Malay versions of the PRISE questionnaires were also retrieved and utilized too.

2.2.9. Assessment of Depression Severity

Different approaches and paradigms have been used in describing the severity of depression in patients as a guide in clinical assessments of the efficacy of treatment phenotype. A decrease in the severity of depression by 50% had commonly been adopted as the hallmark for a clinical response to antidepressant medications [11]. After 2 hospital visits spanning over a period of 2 weeks, the patients were grouped empirically based on established guidelines as either "treatment responders" or "non-responders."

2.2.10. Assessment of Adverse Effect

The patients were grouped empirically based on the presence or absence of AE after 2 weeks of SSRI treatment.

2.3. Processing Urine and Serum

Proton (^{1}H) NMR processing of urine and serum was carried out in accordance with the methods reported in an earlier NMR metabolomics study [12]. Urine samples were thawed and centrifuged at 13000 rpm for 10 minutes. 400 ul of the supernatant was mixed with 200 ul of phosphate buffer solution. The phosphate buffer solution consisted of 0.1% of 3-trimethylsilylproipionic-2,2,3,3-d4 acidic sodium salt (TSP), which was used as an internal standard. Sodium deuterium oxide (NaOD) was used in adjusting the pH to 7.4. The mixture of urine and phosphate buffer solution was transferred meticulously into a 5 mm NMR tube. Serum samples were also thawed and centrifuged at 1300 rpm for 10 minutes, and 200 ul of supernatant was mixed with 400 ul of saline containing 0.2 % TSP, and the mixture was transferred into 5 mm NMR tubes. All buffers and solutions were prepared using deuterated water (D_2O).

2.4. Metabolic Profiling and Data Acquisition using ^{1}H Nuclear Magnetic Resonance

Spectra for urine samples were acquired on a Varian Unity INOVA 500 MHz spectrometer (Varian Inc, CA), with a frequency of 499.887 MHz. Standard one-dimensional PRESAT was used for the suppression of water peaks. For each sample, 64 scans were conducted within an acquisition time of 193 seconds, a pulse width of 3.75 microseconds, and a relaxation delay of 2.0 seconds. Acquisition of the spectra from serum samples was carried out using the combination of PRESAT and Carr-Purcell-Meibom-Gill (CPMG), which suppresses both water peaks and broad signals from macromolecules. The CPMG spectra were acquired after 128 scans [13].

2.5. Chenomx NMR Spectral Data Reduction

Chenomx NMR suite (Chenomx, Calgary, Canada) was used for metabolite identifications and quantifications. Spectral preprocessing, including autophasing, auto baseline correction, and alignment to TSP signal as the internal reference, was applied to each spectrum separately. In the semi-automated chenomix metabolites identification approach (SACMI), metabolites were assigned to spectra based on the suggestions of metabolites from the Chenomx data base and information from the literature. Processed spectra (0-10 ppm) were segmented into bins of 0.04 ppm using the profiler module. Residual signals of water (4.75-4.85 ppm) and those of urea (5.50-6.00 ppm) were excluded from the analysis. The binned spectral data were properly labelled in excel sheets and imported into Simca-P software (Umetrics, Umea, Sweden) for multivariate data analysis.

2.6. Statistical Data Analysis

Statistical assessment of clinical socio-demographic features and levels of metabolites in serum samples of patients was done using Chi-square test, multinomial logistic regression, or Mann-Whitney U statistics in IBM-SPSS (Chicago, IL) software version 22 for Microsoft® Windows. Multivariate data analysis was performed using Pareto scaling. Principal component analysis (PCA), an unsupervised assessment, was used to highlight the pattern of clustering for the samples. Partial least square discriminant analysis (PLS-DA) and orthogonal projections to latent structures-discriminant-analysis (OPLS-DA) are potent supervised methods that maximize the variation between the different study groups based on the class information. Thus, they enumerate the metabolites involved in the separation. Multivariate analysis was conducted using the SIMCA-P software 14.0. The R2X, R2Y, and Q2 values are common pointers for the robustness of the supervised models. The goodness-of-fit of a supervised model was determined by its R2Y cum and Q2 cum scores. Q2 values assess the predictability of the model (Mahadevan *et al.*, 2008). The loading plot of the built model sheds more light on the tentative identities of the metabolites that discriminate between the different categories of diagnosis (HC, TNMDD, TWMDD) and treatment outcomes of study participants [14]. An iteration permutation test can be conducted to assess the level of fitting for models that were generated [15]. Higher original values of R2 and Q2 compared to those of the permutation test suggest that the model was robust [15].

Table 1. Socio-demographic features of study participants.

-	MDD	Controls	Remarks
Age*(median /range) years	32/18-55	24.50/20-55	Mann Whitney U = 106.500, Z = -2.010, p = 0.044
Gender, n = (male/female)	9/16	3/11	-
Ethnicity, n = Malay/Chinese/Indian	12/8/5	7/3/4	-
Educational status*, n = (basic/tertiary)	14/11	2/12	p = 0.011, OR = 7.632, CI = 1.406-41.488
Family income, n = (low/high)	21/4	9/5	-

Abbreviations: *p ≤ 0.05, OR = Odds ratio, CI= Confidence interval, MDD = major depressive disorder, control = Healthy non-MDD volunteers.

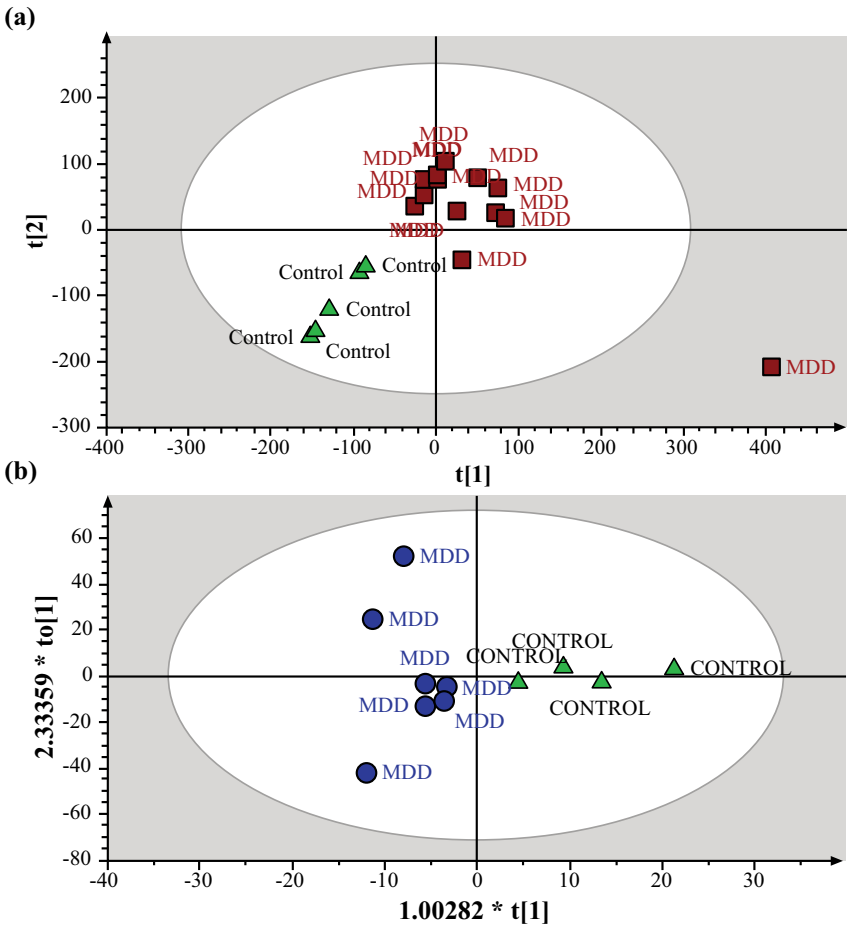

Fig. (1). Multivariate evaluation of the serum samples of healthy volunteers in comparison to the samples of treatment naïve MDD patients (TNMDD labelled as depressed); (**a**) = PLS-DA score plot, (**b**) = OPLS-DA score plot. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

3. RESULTS

3.1. Sociodemographic Features of Study Participants

A total of 8 urine and serum samples each from healthy controls (HC) and twenty-five urine and serum samples each from first episode treatment naïve MDD (TNMDD) patients were recruited for this study. Twenty-two of urine and serum samples from the treatment naïve MDD patients who had received SSRI treatment for 2 weeks (TWMDD) each were made up of ten 2 weeks follow-up samples from some of the initial twenty-five TNMDD patient samples. The remaining twelve urine and serum samples each were from patients who had unsuccessful sampling attempts at baseline (before the commencement of SSRI treatment) with subsequent successful sampling at their 2 weeks follow-up after SSRI treatment onset.

Most of the MDD patients received 54.6% fluvoxamine, 27.3% received sertraline, 13.6% received escitalopram, and 4.5% received fluoxetine. The patients diagnosed and recruited with MDD were of the age range of 18-55 with a median of 32 years, which was significantly (p = 0.044) higher than the median age of the healthy controls, which was 24.5 years (Table **1**). Most of the MDD patients had a basic level of education, while most of the HC attained a

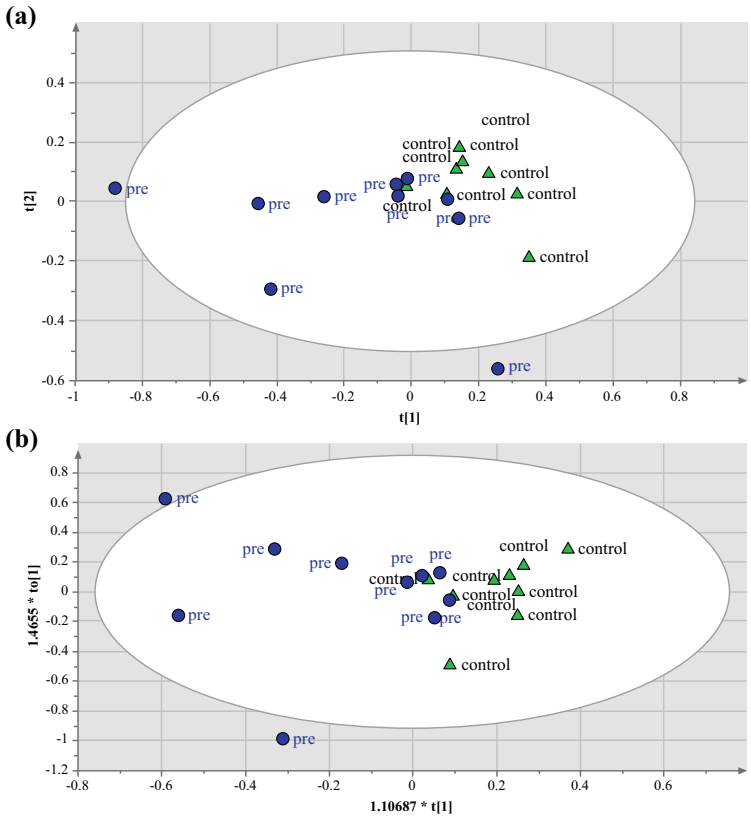

Fig. (2). Multivariate evaluation of the urine samples of healthy controls (control) and samples of treatment naïve MDD patients (TNMDD (labelled as pre)); (**a**) = PLS-DA score plot, (**b**) = OPLS-DA score plot. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

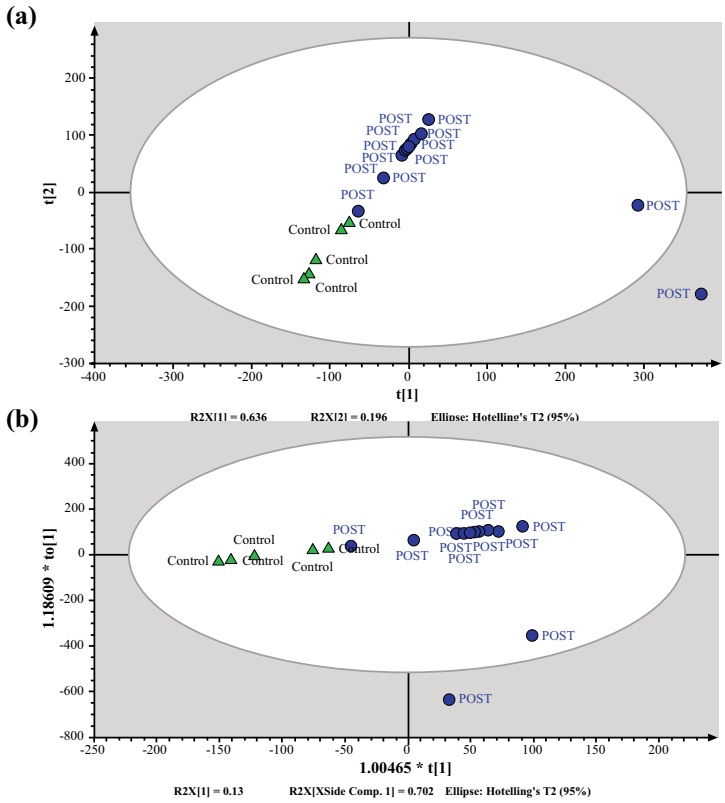

Fig. (3). Multivariate evaluation of the serum samples of treatment naïve MDD patients on SSRI treatment for 2 weeks (TWMDD (labelled as post)) and samples of healthy control (control); (**a**) = PLS-DA score plot, (**b**) = OPLS-DA score plot. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

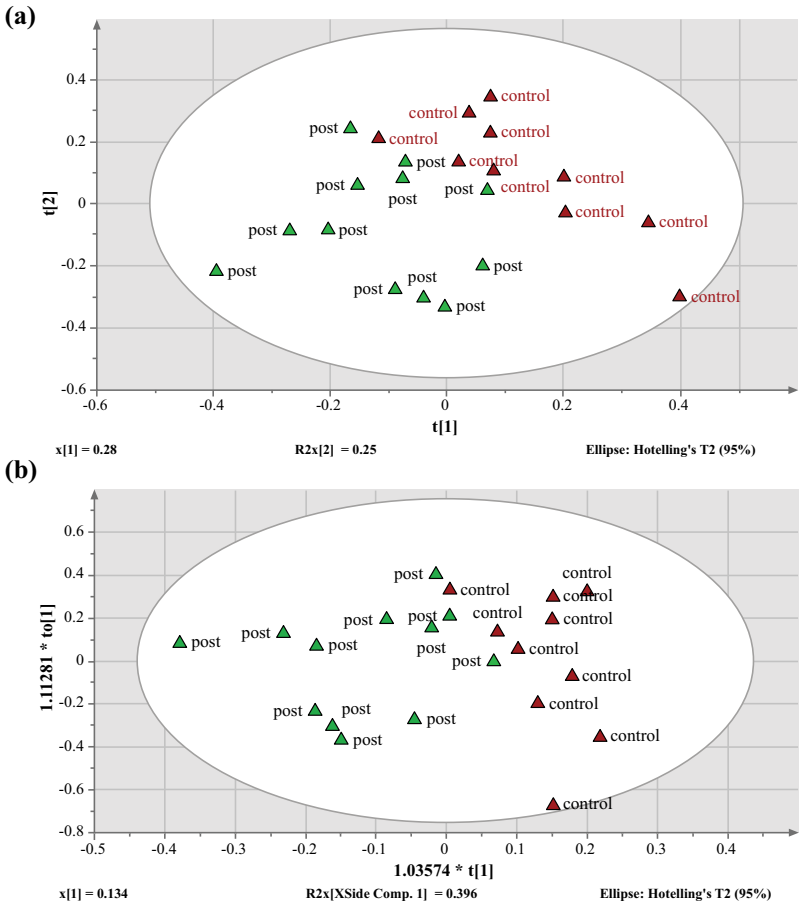

Fig. (4). Multivariate evaluation of the urine samples of treatment naïve major depressive patients on SSRI treatment for 2 weeks (TWMDD (labelled as post) and samples of healthy control (control); (**a**) = PLS-DA score plot, (**b**) = OPLS-DA score plot.

Table 2. Metabolites identified in serum samples using chenomx profiler in study participants.

Metabolites	Chemical Shifts	Remarks
Alanine	1.47 (d), 3.76 (q)	Significant (p = 0.001) in TNMDD *vs.* HC, and in TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.041)
Lactate	1.32 (d), 4.11 (q)	Significant (p = 0.001) in TNMDD *vs.* HC; TNMDD-after 2 weeks SSRI treatment *vs.* HC (p = 0.003); TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.020)
Glycine	3.55 (s)	Significant (p = 0.035) in TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment
Choline	3.19 (s), 3.52(s), 4.06 (s)	Significant (p = 0.004) in TNMDD *vs.* HC; TNMDD-after 2 weeks SSRI treatment *vs.* HC (p = 0.037); TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.02); responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.012)
Acetate	1.91 (s)	Significant (p = 0.006) in TNMDD *vs.* HC;
Betaine	3.88 (s), 3.24 (s)	Responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.038)
Pyruvate	2.35 (s)	Responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.024)
Acetone	2.22 (s)	-
Valine	0.98 (d), 1.040 (d)	Significant (p = 0.012) in TNMDD *vs.* HC; TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.006);
Leucine	3.72 (m)	-
3-Hydroxybutyrate	1.2	-

(Table 2) contd….

Metabolites	Chemical Shifts	Remarks
Creatinine	3.0 (s), 4.1 (s)	Significant (p = 0.009) in TNMDD *vs.* HC; TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.014)
Glutamine	2.10 (s), 2.46 (s)	Significant (p = 0.023) in TNMDD *vs.* HC; TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.014)
GPC	3.200 (s)	Responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.004)
3.26 PPM (TMAO and Betaine)	3.261 (s)	Responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.031)
Glucose	5.23 (s), 3.89 (d), 3.82 (dd), 3.41 (m), 3.40 (m)	Significant (p = 0.00019) in TNMDD *vs.* HC; responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.038)
Phenylalanine	7.32 (d)	-
Fatty acids	0.86 (m)	Significant (p =0.007) in TNMDD *vs.* HC; TNMDD-after 2 weeks SSRI treatment *vs.* HC (p = 0.025)

Abbreviations: S: singlet, d: doublet, ppm: part per million.

Table 3. Metabolites identified in urine samples using chenomx profiler in study participants.

Metabolites	Chemical Shifts (ppm)	Remarks
Imidazole	8.18 (s), 7.28 (s)	-
Formate	8.4 (s)	TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.039); responder *vs.* non-responder to 2 weeks SSRI treatment (p = 0.035)
Hippurate	7.821 (s), 7.619 (s), 7.531 (s)	TNMDD *vs.* TNMDD-after 2 weeks SSRI treatment (p = 0.014);
N-phenylacetylglycine (NPAG)	3.666 (s), 3.774 (d), 7.349 (m) 7.4080 (m) 7.98 (s)	-
Creatinine phosphate	3.950 (s) and 3.000 (s)	-
Glycolate	3.95178 (s)	-
Betaine	3.252 (s)	-
Glycine	3.545 (s)	-
O-phosphocholine	4.166 (dd), 3.637 (t), 3.215 (s)	Significant (p = 0.041) in TNMDD *vs.* HC
Methylmalonate	3.119 (q) 1.216 (d)	-
Trimethylamine-	2.892 (s)	-
Alanine	1.46 (d)	-
Glucose	3.820 (dd) 5.226 (d)	-
Creatinine Citrate	4.043 (s) 2.52 (d), 2.7(d)	Significant (p = 0.041) in TNMDD *vs.* HC
3-Hydroxybutyric acid	2.397 (m)	-
N-acetylglycine	2.028 (s)	-
N-acetylglutamine	2.235 (t)	-
3-methylhistidine	3.66 (s)	-
Phenylacetate	3.545 (s)	-
Methyl malonate	1.230 (s)	-
Leucine	0.948(s)	-
Valine	2.282(m)	-

Abbreviations: S: singlet, d: doublet, ppm: part per million

tertiary level of education and the difference in the educational levels attained by the study participants was statistically significant (p = 0.011). There were more female participants in the current study compared to males, and the study sample was multiethnic. Nevertheless, these differences are not statistically significant (Table **1**).

3.2. Statistical Determination of MDD Diagnostic Metabolites in Serum and Urine

Multivariate data analysis results were generated *via* score plots, loading columns, and variable importance in projection (VIP) in this study, and they facilitated the identification of metabolites that discriminates samples from different phenotypes (Figs. **1**-**4**).

Metabolites identified from urinary and serum spectra of samples from study participants were identified in reference to similar spectra from the HMD-database (Tables **2**, **3** and Fig. (**5**-**7**).

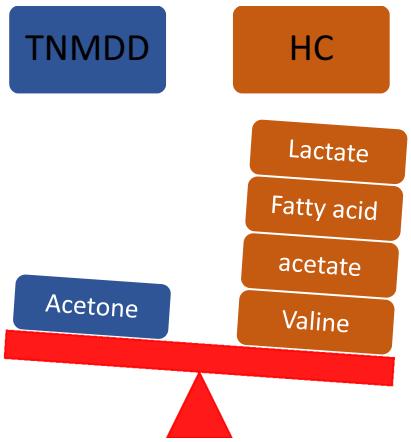

Fig. (5). Schematic representation of the key metabolites differentiating between Treatment Naïve MDD (TNMDD) patients and Healthy controls (HC). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

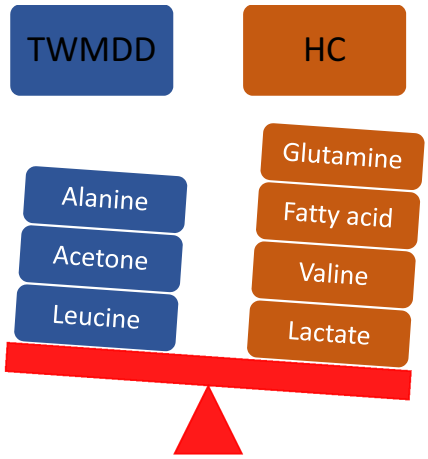

Fig. (6). Schematic representation for the key metabolites distinguishing the serum samples of Treatment Naïve MDD on SSRI treatment for 2 weeks (TWMDD) from those of health control (HC). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

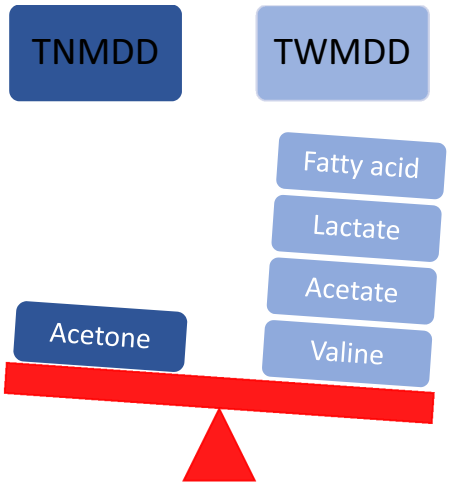

Fig. (7). Schematic representation for the key metabolites distinguishing the serum samples of Treatment Naïve MDD on SSRI treatment for 2 weeks (TWMDD) from those of Treatment Naïve MDD (TNMDD). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

In addition, the metabolites involved in the separation of samples from contrasting phenotypes, including those with high VIP scores and associated levels of reliability and predictability, were highlighted in this study (Tables **4**-**7**).

Serum metabolites in TNMDD patients, distinguishing them from HC, were observed to include glucose, choline, and acetone when OPLS-DA or PLS-DA models were used for the evaluation (Table **4**). Betaine and leucine were additional metabolites identified when the evaluation was done using the PLS-DA model (Table **4**). Samples from HC had fatty acid, valine, lactate, alanine, acetate, glutamine, pyruvate, creatinine, choline, GPC, TMAO, betaine, and glucose metabolites when evaluated using the PLS-DA or OPLS-DA models (Table **4**). Leucine was an additional metabolite identified when the evaluation was done using the OPLS-DA model; GPC was also an additional metabolite that was identified in the samples from HC when the evaluation was done using the PLS-DA model (Table **4**). Lactate, glucose, alanine, valine, fatty acid, choline, glutamine, creatinine significantly contributed to the separation of samples from HC and TNMDD patients in the multivariate (OPLS-DA or PLS-DA) and univariate models. Acetate metabolite was an additional metabolite involved in the separation of samples from TNMMDD and HC phenotypes when the evaluation was done using PLS-DA multivariate or univariate Mann-Whitney U statistics. Acetone was also identified as an additional metabolite only when the evaluation was done using the PLS-DA or OPLS-DA models (Tables **2** and **4**).

Urinary metabolites identified in TNMDD patients relative to HC include creatinine, NPAG and phosphocholine when the evaluation was done using the PLS-DA or the OPLS-DA model (Table **5**). N-acetylgylcine, N-acetylglutamate, methylmalonate and betaine were additional metabolites identified when the evaluation was done using the OPLS-DA model, while Imidazole was an additional metabolite identified when the evaluation was done using the PLS-DA model (Table **5**). Samples from HC were observed to

Table 4. Metabolites involved in the separation of serum samples from patients with different phenotypes of depression and its treatment outcome.

Samples Involved	Phenotypes	Metabolites Identified using OPLS-DA or PLS-DA	Additional Metabolites	VIP Metabolites
TNMDD *vs.* HC	TNMDD	Glucose, acetone, choline	Betaine, leucine (PLS-DA)	Acetone, lactate, glucose, alanine, valine, fatty acid, choline, glutamine, creatinine (OPLS- & PLS-DA); Acetate (OPLS-DA)
	HC	Fatty acid, valine, lactate, alanine, acetate, glutamine, pyruvate, creatinine, choline, GPC, TMAO, betaine, glucose,	GPC (PLS-DA); Leucine (OPLS-DA)	
TWMDD-2 weeks after SSRI treatment *vs.* HC	HC	Fatty acid, valine, lactate, alanine, glutamine, creatinine, TMAO, betaine, choline, lactate	-	Acetone, lactate, leucine, glucose, alanine, choline (OPLS- & PLS-DA); Glycine, betaine, acetate (PLS-DA); fatty acid (OPLS-DA)
	TWMDD-2 weeks after SSRI treatment	Glucose, alanine, betaine, leucine, choline, pyruvate, acetone, acetate	Glutamine, glycine, GPC (PLS-DA)	
TNMDD (Pre) vs. TWMDD-2 weeks after SSRI treatment (post)	TNMDD (Pre)	Glucose, acetone	-	Acetone, lactate, glucose, glycine, leucine, choline, GPC, alanine, valine, betaine, TMAO (OPLS- & PLS-DA), pyruvate (PLS-DA), fatty acid, glutamine, creatinine (OPLS-DA)
	TWMDD-2 weeks after SSRI treatment (post)	Lactate, choline, creatinine, glucose, alanine, leucine, glycine, TMAO, glutamine, pyruvate, acetate, valine, fatty acid, betaine	-	
Non-responder *vs.* responder	Non-responder (nr)	Choline, creatinine, glucose, betaine, alanine, leucine, glycine, TMAO, betaine, GPC.	Alanine (PLS-DA), fatty acid (OPLS-DA)	Acetone, pyruvate, choline, GPC, glucose, acetate, TMAO, betaine, glycine, lactate (OPLS- & PLS-DA), Creatinine, valine (PLS-DA), leucine (OPLS-DA)
	Responder (r)	Lactate, creatinine, glutamine, pyruvate, acetone, acetate, alanine, valine, fatty acid.	-	
Adverse effects (se) vs. No adverse effect (nse)	Adverse effect (AE)	Lactate, acetone	Glucose, glutamine (OPLS-DA)	Acetone, lactate, acetate, choline, alanine, pyruvate, glycine, glucose, leucine, betaine, TMAO, GPC (OPLS- & PLS-DA), creatinine, valine (PLS-DA)
	No adverse effect (NAE)	Fatty acid, valine, alanine, acetate, glutamine, creatinine, choline, GPC, TMAO, betaine, glucose, glycine, leucine	Pyruvate (PLS-DA)	

Abbreviations: TNMDD = treatment naïve major depressive disorder, TWMDD = Treatment naïve MDD patients 2 weeks after SSRI treatment, PLSDA = partial least square-discriminant analysis, OPLSDA = orthogonal partial least square-discriminant analysis, VIP = variable important projections.

contain alanine, formate, 3-hydroxybutyrate, citrate, trimethylamine, creatinine, glycine, phenylacetate, phosphocholine, glucose, 3-methylhistidine, NPAG, and Hippurate when the evaluation was done using the PLS-DA or the OPLS-DA model. Nevertheless, in the PLS-DA model, N-acetylglycine, N-acetylglutamate, methylmalonate, and betaine (PLS-DA) were additional metabolites observed in the samples of HC. (Table **5**). Phosphocholine and citrate metabolites contributed significantly to the multivariate (OPLS-DA and PLS-DA) and univariate separation models for urine samples of TNMDD and HC in this study (Table **3** and **5**). Creatine, creatinine, 3-methylhistidine, phosphocholine, citrate, hippurate, phenylacetate, glucose, and NPAG were metabolites that were significantly involved in the multivariate model (PLS- & OPLS-DA) for the separation of the urine samples between the TNMDD and the HC phenotypes (Table **5**). Glycine, when the evaluation was done using the OPLS-DA model, as well as methylmalonate and/or betaine when the evaluation was done using the PLS-DA model, were additional urinary metabolites for the separation of TNMDD from HC phenotypes (Table **5**).

Serum metabolites in samples of TWMDD when using either PLS-DA or OPLS-DA models during evaluation included glucose, alanine, betaine, leucine, choline, pyruvate, acetone, and acetate. Glutamine, glycine, and GPC were additional metabolites observed in these serum samples following an evaluation using the PLS-DA model (Table **4**). The samples from participants with the HC phenotypes were observed to contain fatty acid, valine, alanine, glutamine,

Table 5. Metabolites involved in the separation of urine samples from patients with different phenotypes of depression and its treatment outcome.

Samples Involved	Phenotypes	Metabolites Identified using OPLS-DA or PLS-DA	Additional Metabolites	VIP Metabolites
TNMDD *vs.* HC	TNMDD	NPAG, creatine, phosphocholine	N-acetylgylcine, N-acetylglutamate,methylmalonate, betaine (OPLS-DA); Imidazole (PLSDA)	Creatine, creatinine, 3-methylhistidine, phosphocholine, citrate, hippurate, phenylacetate, glucose, NPAG, (PLS- & OPLS-DA), glycine (OPLS-DA), methylmalonate, betaine (PLS-DA)
	HC	Alanine, formate, 3-hydroxybutyrate, citrate, trimethylamine, creatinine, glycine, phenylacetate, phosphocholine, glucose, 3-methylhistidine, NPAG, Hippurate	N-acetylgylcine, N-acetylglutamate, methylmalonate, betaine (PLS-DA)	
TWMDD- 2 weeks after SSRI treatment *vs.* HC	HC	Creatinine, glucose, NPAG, 3-methylhistidine, phenylacetate, glycine, phosphocholine, methylmalonate, trimethylamine, alanine, N-acetylglutamate, N-acetylglycine, 3-hydroxybutyrate, citrate	Hippurate (OPLS-DA)	Creatine, creatinine, 3-methylhistidine, phosphocholine, citrate, betaine, glycine, phenylacetate, glucose, NPAG, N-acetylglutamate, N-acetylglycine, methylmalonate, 3-hydroxybutyrate (PLS- & OPLS-DA), hippurate, imidazole, trimethylamine, alanine, (PLS-DA)
	TWMDD-2 weeks after SSRI treatment (Post)	Formate, imidazole, NPAG, creatine, betaine	Hippurate (PLS-DA)	
TNMDD (Pre) *vs.* TWMDD-2 weeks after SSRI treatment (Post)	TNMDD (Pre)	-	-	Creatine, creatinine, betaine, NPAG, citrate, phosphocholine, glucose, hippurate, 3-methylhistidine, N-acetylglutamate, phenylacetate, methylmalonate, alanine (OPLS- & PLS-DA), imidazole, N-acetylglycine (PLS-DA)
	TWMDD-2 weeks after SSRI treatment (Post)	Formate, imidazole, NPAG, Hippurate, phosphocholine, creatinine, creatine, glucose, 3-methylhistidine, alanine, N-acetylglutarate, N-acetylglycine, phenylacetate, phosphocholine, NPAG, glycine, methylmalonate, 3-hydroxybutyrate, glucose	Betaine, citrate, 3-hydroxybutyrate, alanine	
Responder (r) *vs.* non-responder	Responders (r)	Hippurate, phospholine, creatinine, glucose, NPAG, 3-methylhistidine, phenylacetate, glycine, betaine, methylmalonate, trimethylamine, citrate, 3- hydroxybutyrate, N-acetylglutamate	N-acetylglycine (PLS-DA)	Creatinine,creatine, 3-methylhistidine, Hippurate, phosphocholine, Hippurate (PLS- & OPLS-DA); Citrate, NPAG, betaine, methylmalonate, glucose, phenylacetate, N-acetylglutamate (PLS-DA)
-	Non-responders (nr)	NPAG, formate, imidazole, creatinine, alanine, creatine	Trimethylamine (OPLS-DA)	-
Adverse effects (se) *vs.* No adverse effect (nse)	Adverse effects (se)	Phosphocholine, creatine, glucose, glycine, creatinine, trimethylamine, N-acetylglycine, methylmalonate	Betaine, citrate, 3-hydroxybutyrate, alanine, hippurate	Phosphocholine, 3-methylhistidine, NPAG, betaine, glucose, glycine, citrate (OPLS- & PLS-DA); Creatine, creatinine(PLS-DA)
	No adverse effect (nse)	Formate, Imidazole, NPAG, 3-methylhistidine, phosphocholine, phenylacetate	Alanine, 3-hudroxybutyrate, citrate, methylmalonate, betaine, creatinine, hippurate.	

Abbreviations: TNMDD = treatment naïve major depressive disorder, PLSDA = partial least square-discriminant analysis, OPLSDA = orthogonal partial least square-discriminant analysis, VIP = variable important projections.

creatinine, TMAO, betaine, choline, and lactate. Lactate and choline are metabolites that were significantly responsible for the separation of serum samples of TWMDD from those of HC when the evaluation was done using multivariate (OPLS- & PLS-DA) as well as univariate analysis. Acetone, leucine, glucose, alanine were the key determinant for discrimination when the evaluation was done using multivariate evaluation (OPLS- & PLS-DA) for samples from HC and TWMDD. Glycine, betaine, acetate were the metabolites of interest when the evaluation was done using the PLS-DA

Table 6. The performance of discriminatory models in correctly separating study observations into their respective classes based on metabolites in the serum and urine.

Sample Type	Phenotypes Under Study	Model	Predictive Performance in Score Plot	Fischer's Exact Statistics
Serum samples	Treatment naive MDD patient *vs.* non-depressed volunteers	PLSDA	96.88%	2.4e-006
		OPLSDA	96.88%	2.4e-006
	MDD patient on treatment for 2 weeks *vs.* non-depressed volunteers	PLSDA	95.83%	2.3e-005
		OPLSDA	95.83%	2.3e-005
	MDD patient on treatment for 2 weeks *vs.* treatment naive MDD patients	PLSDA	84.85%	7.5e-005
		OPLSDA	84.85%	7.5e-005
	Treatment response *vs.* non-response to treatment	PLSDA	88.89%	0.0011*
		OPLSDA	88.89%	0.0011*
	Adverse effect *vs.* no adverse effect	PLSDA	94.12%	0.0021*
		OPLSDA	94.12%	0.0021*
Urine samples	TNMDD *vs.* HC	PLSDA	84.21%	0.0024*
		OPLSDA	84.21%	0.0024*
	TNMDD after 2 weeks of SSRI *vs.* HC	PLSDA	90.91%	0.00019*
		OPLSDA	90.91%	0.00019*
	TNMDD *vs.* TNMDD after 2 weeks of SSRI	PLSDA	78.57	0.063
		OPLSDA	78.57	0.063
	Responders *vs.* non-responders to SSRI treatment for 2 weeks	PLSDA	85.71%	0.015*
		OPLSDA	85.71%	0.015*
	Adverse effect *vs.* no adverse effect to SSRI treatment for 2 weeks	PLSDA	83.33%	0.045*
		OPLSDA	83.33%	0.045*

Abbreviations: PLSDA = Partial Least Square Discriminant Analysis, OPLSDA = Orthogonal Projections to Latent Structures-Discriminant Analysis, N = sample size, A = number of principal components, R2Y = measure of the reliability of the model, Q2Y = measure of predictability of model, pCV ANOVA* = statistics for cross-validation.

model, while fatty acid was the metabolite of interest when the evaluation was done using the OPLS-DA model or univariate statistics for the discrimination of samples from TWMDD patients from those of HC (Table **4**).

Urinary imidazole, NPAG, creatine, betaine, and formate were increased in samples of patients with TWMDD phenotypes when compared to samples from HC, and this was irrespective of the model (PLS-DA or OPLS-DA) used for evaluation (Table **5**). Methylmalonate, creatinine, glucose, NPAG, 3-methylhistidine, phenylacetate, glycine, phosphocholine, trimethylamine, alanine, N-acetylglutamate, N-acetylglycine, 3-hydroxybutyrate, and citrate were present in the samples of HC when evaluations were done with either OPLS-DA or PLS-DA models (Table **5**). Hippurate was an additional metabolite identified in samples from HC when the evaluation was done using OPLS-DA. It was also observed in samples from TWMDD when the evaluation was done using the PLS-DA model. Creatine, creatinine, 3-methylhistidine, phosphocholine, citrate, betaine, glycine, phenylacetate, glucose, NPAG, N-acetylglutamate, N-acetylglycine, methylmalonate and 3-hydroxybutyrate contributed significantly in the separation of samples when the evaluation was done using either PLS-DA or OPLS-DA model. Hippurate, imidazole, trimethylamine, and alanine were additional metabolites significantly involved in the separation of samples when the evaluation was done using the PLS-DA model (Table **5**).

3.3. Statistical Determination of SSRI Treatment Prognostic Metabolites in Serum and Urine

In both OPLS-DA and PLS-DA models, serum levels of lactate, glutamine, pyruvate, acetone, acetate, alanine, valine, fatty acid, and creatinine were relatively higher in the patients who responded to SSRI treatment compared to those who did not. Choline, glucose, betaine, alanine, leucine, glycine, TMAO, betaine, GPC, and creatinine were observed to have increased in the samples of patients who did not respond to 2 weeks SSRI treatment when the evaluation was done using either PLS-DA or OPLS-DA model. Acetone, pyruvic acid, choline, GPC, glucose, acetate, TMAO, glycine, betaine, and lactate contributed significantly to the separation of samples based on treatment response phenotypes, as observed in the PLS-DA or OPLS-DA model. Additional

Table 7. Validation of the models for the separation of the metabolites in urine and serum samples.

Phenotype Under Study	Samples	Models	N	A	R2Y	Q2Y	pcv ANOVA
TNMDD *vs*. HC	Serum	PLS-DA	32	2	0.767	0.595	6.24429e-005*
		OPLSDA	32	1+1+0	0.767	0.599	3.9984e-005*
	Urine	PLS-DA	19	2	0.485	0.024	0.9665
		OPLSDA	19	1+1+0	0.485	0.173	0.584
TNMDD on SSRI treatment for 2 weeks *vs*. HC	Serum	PLS-DA	24	2	0.832	0.662	0.00055*
	-	OPLSDA	24	1+1+0	0.832	0.595	0.00124*
	Urine	PLS-DA	22	2	0.628	0.450	0.0306*
	-	OPLSDA	22	1+1+0	0.628	0.425	0.0417*
TNMDD *vs*. TNMDD on SSRI treatment for 2 weeks	Serum	PLS-DA	33	2	0.493	0.303	0.0205
		OPLSDA	33	1+1+ 0	0.493	0.309	0.0301
	Urine	PLS-DA	14	2	0.597	0.157	0.7032
		OPLSDA	14	1+1+0	0.597	0.349	0.3734
Responder *vs*. non-responders to SSRI treatment for 2 weeks	Serum	PLS-DA	18	2	0.671	0.380	0.1578
		OPLSDA	18	1+1+0	0.671	0.333	0.2268
	Urine	PLS-DA	14	2	0.574	0.161	0.500
		OPLSDA	14	1+1+0	0.574	0.125	1.000
Adverse effect *vs*. no-adverse effect to SSRI treatment for 2 weeks	Serum	PLS-DA	17	2	0.626	0.294	0.2055
		OPLSDA	17	1+2+0	0.72	0.281	0.6890
	Urine	PLS-DA	12	2	0.464	-0.21	1.000
		OPLSDA	12	1+1+0	0.464	-0.164	1.000

Abbreviations: PLSDA = Partial Least Square Discriminant Analysis, OPLSDA = Orthogonal Projections to Latent Structures- Discriminant Analysis, N = sample size, A = number of principal components, R2Y = measure of the reliability of the model, Q2Y = measure of predictability of model, pCV ANOVA*= statistics for cross-validation.

metabolites, such as valine and creatinine, also contributed to the separation of samples in evaluation using the PLS-DA model, while leucine contributed to the separation of the samples for evaluations using the OPLS-DA model. Univariate statistical evaluation revealed a significant increase of betaine ($p = 0.038$), choline ($p = 0.012$), glucose ($p = 0.038$), 3.26 ppm (TMAO and betaine) ($p = 0.031$) and GPC ($p = 0.004$) in samples of patients who failed to respond to the treatment. It also showed an increased level of pyruvate ($p = 0.024$) in the samples of patients who responded to treatment (Table **2**). Therefore, choline, glucose, TMAO, betaine, GPC, and pyruvate metabolites were observed to be significantly involved in the separation of samples from patients with phenotypes responding and/or not responding to SSRI treatment regardless of the method of statistical evaluation applied.

NPAG, hippurate, phospholine, creatinine, glucose, NPAG, 3-methylhistidine, phenylacetate, glycine, betaine, methylmalonate, trimethylamine, citrate, 3- hydroxybutyrate, and N-acetylglutamate were increased in urinary samples from patients who responded to SSRI treatment when the evaluation was done using OPLS-DA or PLS-DA model (Table **5**). N-acetylglycine was an additional metabolite that was identified in the sample obtained from responders to SSRI treatment when the evaluation was done using the PLS-DA model (Table **5**). NPAG, formate, imidazole, creatinine, alanine, and creatine were metabolites that increased in the urine samples obtained from patients with the non-responder phenotype when the evaluation was done using OPLS-DA or PLS-DA model (Table **5**). Trimethylamine was an additional metabolite identified when the evaluation was done using the OPLS-DA model (Table **5**). Creatinine, creatine, 3-methylhistidine, phosphocholine and hippurate were metabolites that were significantly involved in the separation of urinary samples of both responders and non-responders to SSRI treatment in this study when the evaluation was done using either PLS-DA or OPLS-DA model (Table **5**). Citrate, NPAG, betaine, methylmalonate, glucose, phenylacetate, N-acetylglutamate were additional metabolites that were involved in the separation for the samples when the evaluation was done using the PLS-DA model (Table **5**). Univariate statistical evaluation revealed a significantly increased level of formate ($p = 0.035$) in the samples obtained from patients with the phenotype non-responsive to 2 weeks of SSRI treatment compared to those of the responders to treatment (Table **3**).

Serum fatty acid, valine, alanine, acetate, creatinine, choline, GPC, TMAO, betaine, glucose, glycine, leucine, and glutamine were identified among patients with no-adverse effects when separation was based on AEs phenotypes, irrespective of the evaluation model used (OPLS-DA or PLS-DA) (Table **4**). Pyruvate was an additional metabolite identified when the evaluation was done using the PLS-DA model only (Table **4**). Lactate and acetone were increased in the serum samples of patients with AEs when the evaluation was done using the PLS-DA or OPLS-DA model (Table **4**). Glucose and glutamine were also observed to have increased in the samples from patients with AEs when the evaluation was done using the OPLS-DA model. Acetone, lactate, acetate, choline, alanine, pyruvate, glycine, glucose, betaine, TMAO, GPC, and leucine contributed significantly to the separation of samples based on phenotypes of AEs when the evaluation was done using the PLS-DA or OPLS-DA model (Table 2). In addition, creatinine and valine also contributed significantly to the separation of these samples when the evaluation was done using the PLS-DA model (Table **2**).

The evaluation using the OPLS-DA or PLS-DA model revealed that phosphocholine, creatine, glucose, glycine, creatinine, trimethylamine, N-acetylglycine, and methylmalonate were increased in urinary samples obtained from patients reporting at least one AE to treatment (Table **5**). The evaluation using the PLS-DA revealed that betaine, citrate, 3-hydroxybutyrate, alanine and hippurate were additional metabolites that were increased in urinary samples from patients reporting an AE to treatment. Samples from patients without any AEs were observed to have increased formate, imidazole, NPAG, 3-methylhistidine, phosphocholine, and phenylacetate when the evaluation was done using the OPLS-DA or PLS-DA model. Alanine, 3-hydroxybutyrate, citrate, methylmalonate, betaine, creatinine, hippurate were additional metabolites identified when the PLS-DA model was used (Table **5**). Phosphocholine, 3-methylhistidine, NPAG, betaine, glucose, glycine, and citrate are metabolites that were significantly involved in the separation of the urinary samples from patients with the phenotypes of AEs and/or No AEs when the evaluation was done using the OPLS-DA or the PLS-DA model (Table **5**). Creatinine and creatine were additional metabolites that significantly contributed to the separation of these samples when the evaluation was done using the PLS-DA model.

3.4. Validation of MDD Diagnostic Model

Diagnostic models of serum samples of TNMDD (before and after 2 weeks of SSRI treatment) and HC were observed to have significant cross-validation outcomes for the OPLS-DA model and optimal permutation outcomes for the PLS-DA model in this study (Tables **6**, **7**).

3.5. Validation of the MDD Treatment Outcome Prognostic Models

All the prognostic models for AEs and/or efficacy phenotypes were observed to have non-significant levels of cross-validations and poor permutation outcomes in this study (Tables **6**, **7**), and thus, were considered to have little or no validity.

4. DISCUSSION

In the literature, urine samples obtained from MDD patients had high levels of α-ketoglutarate, TMAO, indoxylsulphate, m-hydroxyphenylacetate, malonate, 3-hydroxyphenylacetic acid, N-methylnicotinamide and oxalacetate. The samples had low levels of nicotinate, p-Hydroxyphenylacetate, sucrose, alanine, taurine, choline, citrate, hydroxylamine, myristic acid, formate, isobutyrate, palmitic acid, lactate and glycine [16]. The metabolite changes that differentiate urine samples of HC from those of patients with moderate MDD include reductions in TMAO, N-Methylnicotinamide, acetone, choline, malonate and glyceroylphosphocholine (GPC) as well as increases in the levels of fructose, nicotinate, citrate, isobutyrate, ribose, vanillic acid, sorbitol and azelaic acid [16]. Statistical evaluations have revealed that citrate, choline, azelaic, N-methylnicotinamide metabolites are the most vital metabolites identified in samples of HC patients relative to those patients with moderate MDD. Citrate, 3-hydroxyphenylacetic acid, palmitic acid, and lactate metabolites were identified in the samples of HC and profoundly played a vital role in discriminating severe MDD from HC [16]. In the current study, samples of HC in relation to those obtained from TNMMD patients contain alanine, formate, 3-hydroxybutyrate, trimethylamine, creatinine, phenylacetate, phosphocholine, glucose, 3-methylhistidine, NPAG, hippurate. glycine, citrate and formate metabolites. The last 3 metabolites (glycine, citrate, and formate) were the same metabolites identified in the literature. The significant observation for lower levels of citrate and phosphocholine in TNMDD patients' samples compared to HC, irrespective of the model used during evaluation, was also similar to the findings in the literature. The differences observed in the direction of metabolites concentration, the specificities for chemical moieties identified in this study as well as the literature findings may be related to the severity of MDD of participants in the literature study, which was not considered in the current study. In addition, the inability of the current study to identify other metabolites, such as azelaic and N-methylnicotinamide for moderate MDD or 3-hydroxyphenylacetic acid, palmitic acid, and lactate for severe MDD reported in the literature may be attributed to the fact that the severity of TNMDD was not factored in determining the samples for analysis. Therefore, these significant metabolites for the separation of HC and TNMDD samples identified in the literature may be specific for assessments based on MDD severity, while the separating metabolites observed in the current study are not based on MDD severity.

In the current study, serum samples from TNMDD patients indicate relatively high levels of acetone, choline, and glucose as well as low levels of glutamine, lactate, and pyruvate. High levels of glutamine (in addition to glycine and serine) had been reported from the serum evaluation in postmortem studies of MDD patients [17-20]. Some studies cited in the literature have not indicated any differences in plasma glutamate, glycine, and serine of MDD patients when compared to those of HC [17, 21, 22]. In addition, another study using column-switching high-performance liquid chromatography (HPLC) system for evaluating plasma amino acid levels among MDD patients has revealed an increase in the level of serine amino acid, a decrease in glycine, glutamate, and glutamine [21, 23]. In the current study, failures to detectincreases in serum glycine, serine, glutamate in the samples of

TNMDD patients, as reported in earlier studies, and the identification of decreased glutamine in serum of patients with TNMDD phenotype could be due to their roles in the discrimination of samples remaining contentious. Glutamine is one of the serum metabolites that significantly determined the separation of TNMDD from HC samples in this study. This suggests high reliability and predictability in discriminating between serum samples from TNMDD and those from HC. Nonetheless, there are other significant metabolites that play a key role in separating TNMDD from HC serum samples, such as lactate, glucose, alanine, valine, fatty acid, creatinine, acetate and choline in this study. An earlier urine metabolomic study had demonstrated that lactate and choline, in tandem with other metabolites, significantly contributed to the separation of MDD from HC samples. The difference in study design, especially as it relates to the recruitment of samples in the earlier study based on the severity of MDD, may account for the difference observed in the findings of the current and earlier studies. In the current study, acetone, lactate, glucose, alanine, and choline are metabolites that significantly differentiated serum samples of patients with the phenotypes of TNMDD and/or TWMDD from those with HC phenotype. Nevertheless, fatty acid, valine, glutamine, creatinine have proven to be additional significant metabolites for the separation models of TNMDD from HC, while leucine acts as an additional metabolite in the separation model involving samples of TWMDD and HC. Therefore, from the foregoing discussion, it is evident that the study design, tools, and techniques used in ascertaining metabolites may play vital roles in the specific differences of the array of metabolites reported to be involved in the diagnosis of MDD. Additionally, key metabolites involved in the discrimination of samples may continue to be highlighted regardless of the evaluation models.

Decreased urinary creatinine, proline, betaine, hippurate, PAG, m-HPPA, formate, acetate, propionate, DMA, MA and increased levels of O-acetyl and N-acetyl glycoproteins following treatment with SSRI have been reported in the literature. Low levels of glycine, glutamic acid and high levels of asparagine, aspartic acid, and hydroxylamine have also been associated with a better SSRI treatment outcome. A key source of glycine is the activity of a hydroxymethyltransferase enzyme on serine thus, lower levels of serine may also be encountered in MDD patients responding to SSRI treatment [21, 23]. In a liquid chromatography–tandem mass spectrometry (LC-MS/MS) study, metabolite changes following SSRI treatment in MDD patients revealed that responders to SSRI had a significantly high baseline of alpha-aminobutyric acid (ABA) level and a marked reduction following treatment response. The level of glutamic acid among the treatment responders was significantly decreased, thus significantly altering the ratio of glutamine to glutamic acid ($p = 0.014$) [24]. Decrease in the levels of ribose, trehalose, and cystine commonly followed SSRI treatment with an improvement in depression symptoms. Attenuations of the levels of branched-chain amino acids (valine, leucine and isoleucine), linoleic, palmitic, oleic, palmitoleicheptadecanoic acids, glycerol, ornithine, citrulline, and xanthine were correlated with an SSRI reduction of depression severity. The correlations of decreased levels of linoleic, arachidonic, palmitic acids, ornithine, and glycerol were more evident after 4 weeks of SSRI treatment as appraised using a GCMS system. Increased levels of cysteine, lactic acid and pseudouridine, arachidonic acid and alpha-ketoglutarate were also correlated with SSRI-related reductions in the severity of depression [24-29]. A validated liquid chromatography electrochemical array (LCECA) platform revealed changes in the concentration of 5HT, HPAC, KYN, 5-MTPM, LD, HGA, 4-HPLA and HGA in the plasma of MDD patients treated with SSRI. These metabolites are members of the methoxyindole and kynurenine (KYN) branches of the tryptophan pathway that were associated with SSRI treatment response [30]. The ratio of the KYN/MEL and 3-OHKY/MEL were significantly decreased in post-treatment plasma samples of MDD patients who had a significant treatment response to SSRI compared to their pre-treatment levels. This implies that the methoxyindole branch of the tryptophan pathway was relatively more active in these patients compared to the KYN branch [30, 31]. The activity of the KYN pathway,which was promoted by pro-inflammatory cytokines that activate the indoleamine 2,3-dioxygenase (IDO) responsible for catalysing the metabolism of tryptophan into KYN as well as increasing activation of the KYN pathway, play a substantial role in the reductions of 5-HT synthesis levels in both MDD patients and individuals who develop depression following cytokine administration [32, 33]. An evaluation of metabolites in remitted MDD (rMDD) patients revealed a higher TYR/4HPLA and low 5HIAA/KYN, TYRA, HVA/ MHPG, HVA/TYR ratios. This means that more tryptophan was shunted towards the KYN pathway and not the 5-HT and/or the norephinephrine synthesis. The pro-inflammatory state of depression appears to persist among rMDD patients despite the resolution of depressive symptoms [34]. Reductions of IL-6 and TNF–alpha activities following SSRI treatment have been reported, and these were pro-inflammatory factors that were most consistently associated with MDD [35, 36]. In the current study, the different approaches to evaluating SSRI treatment outcomes using either the PLS-DA or OPLS-DA model nominally identified various metabolites, as reported in the results section. However, none of the separation models has survived the different statistical tests for reliability and predictability. The failure of the reliability and predictability tests of the prognostic models in the current study could be associated with either short durations of SSRI treatment, the diversity in the SSRI medications prescribed to the patients, or the limited sample size in our study.

4.1. Putative Metabolism Pathway Implicating Identified Metabolites

In healthy individuals, lactic acid is generated mainly from RBC skin and brain. Kidney and liver activities promote the conversion of lactic acid into carbon dioxide, water, and a substrate for gluconeogenesis. Its production and utilisation with pyruvic acid are in a state of homeostasis [37], which is also due to the aerobic breakdown of pyruvate, which generates NAD+ and acetate, a vital substrate for the Kreb cycle. Oxidation of pyruvate and NADH generated from glucose in glycolysis is hampered in an anaerobic setting. The regeneration of NAD+ by reducing pyruvate to lactate is thus an adaptive process that ensures a continued metabolism in anaerobic settings to ensure the regeneration of NAD+. Aerobic metabolisms of glucose with the generation of lactate and NAD+ have also been elucidated in the

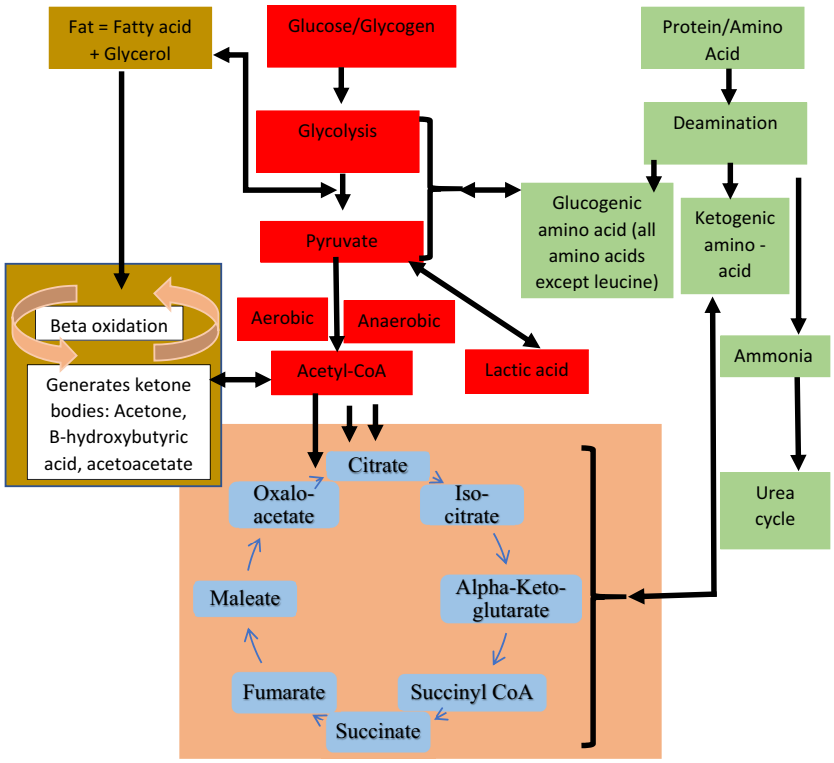

Fig. (8). Pictorial representation of normal-state metabolites status and pathway for metabolism in non-MDD healthy volunteers. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

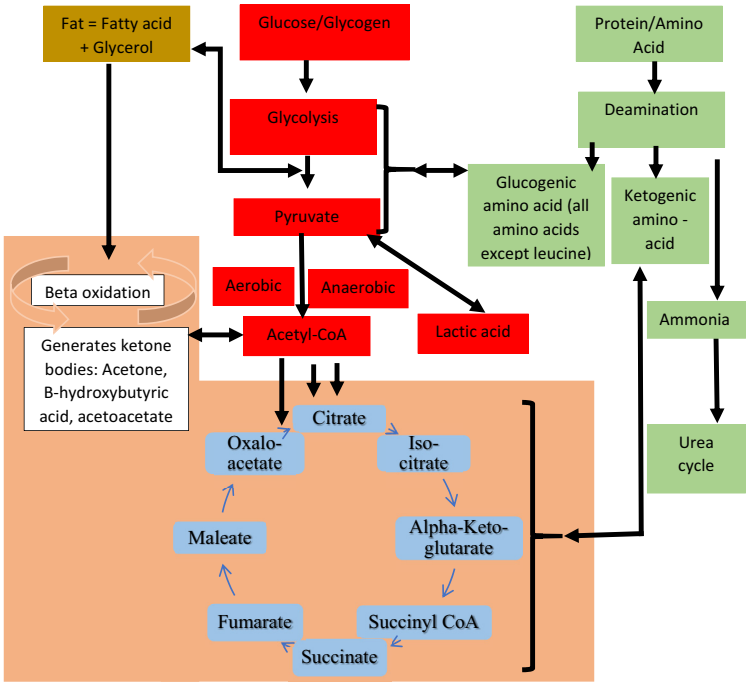

Fig. (9). Pictorial representation of shift of metabolites status among MDD patients (both treatment naive and those on treatments for 2 weeks). There is a prevalence for fatty acid-beta oxidation pathway, thus accounting for increased levels of its metabolic products. See the highlighted segment of the diagram. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

literature [38]. Energy generation from oxidation of succinate, citrate, maleate and glutarate has also been reported. Formations of succinate from fumarate and oxaloacetic acid through oxidative and reductive processes are key features of the Kreb cycle. The generation of succinate is enhanced by the activity of malonate, a key inhibitor of succinate dehydrogenase. NAD-linked malate dehydrogenase oxidizes malonic acid to oxaloacetic acid with the generation of energy.

Nonetheless, malonic acid is commonly associated with NADP rather than NAD thus, providing an alternative source of reduced NADP for numerous other biosynthetic reactions [39]. Therefore, regardless of the scenario, the generation of energy and reduction factors for biochemical reactions are maintained in health [39]. Disturbances in the energy metabolism of different etiologies, such as mitochondrial dysfunction, have been hypothesized to be key in the development of MDD [40-43]. Mitochondrial dysfunction renders the activity of the Kreb cycle enzymes, including the citrate synthase and the succinate dehydrogenase. SSRI treatment has reportedly been associated with improving the activity levels of these enzymes in preclinical studies [24].

In the current study, the normal levels of lactate and pyruvic acid observed in samples of HC compared to their low levels in TNMDD before and after an SSRI treatment for 2 weeks suggest that the carbohydrate metabolism in depression is low. Increased pyruvate, lactate and other pointers to carbohydrate metabolism are hypothetically envisaged to be encountered among patients with a treatment response efficacy, as observed in this study. Schematic representations of metabolic pathways in non-depressed volunteers and MDD patients have been illustrated in Figs. (**8** and **9**).

CONCLUSION

There were some variabilities in the enumerated metabolites identified in both this study and in the literature, while there are some findings in this study that are consistent with the literature. Statistical validations of discriminatory metabolites were successful for the model of MDD diagnosis using serum samples of TNMDD and TWMDD patients in comparison to samples from HC. The main metabolites underlying these validated discriminatory models included decreased levels of lactate, glucose, glutamine, creatinine, acetate, valine, alanine, fatty acid and increased levels of acetone and choline in TNMDD and TWMDD samples, irrespective of whether an OPLS-DA or a PLS-DA model of evaluation was used. These results were also in line with the statistical results obtained using univariate evaluations of the relative concentrations of the metabolites in various samples. In addition, univariate evaluations have revealed that urinary citrate and phosphocholine metabolites were significantly different in the samples of TNMDD and HC, although the OPLS-DA and PLS-DA models were not validated for reliability and predictability.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the the Medical Research and Ethics Committee (MREC) of Ministry of Health, Malaysia (NMRR-14-688-19696).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/ 10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Informed consent was obtained from the potential participants.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

STROBE checklist is available on the publisher's website along with the published article.

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