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Plasma metabolic biomarkers for discriminating individuals with alcohol use disorders from social drinkers and alcohol-naive subjects

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ABSTRACT

Background: Alcohol use disorders (AUD) is a phase of alcohol misuse in which the drinker consumes excessive amount of alcohol and have a continuous urge to consume alcohol which may lead to various health complications. The current methods of alcohol use disorders diagnosis such as questionnaires and some biomarkers lack specificity and sensitivity. Metabolomics is a novel scientific field which may provide a novel method for the diagnosis of AUD by using a sensitive and specific technique such as nuclear magnetic resonance (NMR).
Methods: A cross sectional study was conducted on three groups: individuals with alcohol use disorders ($n = 30$), social drinkers ($n = 54$) and alcohol-naive controls ($n = 60$). ^{1}H NMR-based metabolomics was used to obtain the metabolic profiles of plasma samples. Data were processed by multivariate principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) followed by univariate and multivariate logistic regressions to produce the best fit-model for discrimination between groups.
Results: The OPLS-DA model was able to distinguish between the AUD group and the other groups with high sensitivity, specificity and accuracy of 64.29%, 98.17% and 91.24% respectively. The logistic regression model identified two biomarkers in plasma (propionic acid and acetic acid) as being significantly associated with alcohol use disorders. The reproducibility of all biomarkers was excellent (0.81–1.0).
Conclusions: The applied plasma metabolomics technique was able to differentiate the metabolites between AUD and the other groups. These metabolites are potential novel biomarkers for diagnosis of alcohol use disorders.

1. Introduction

Alcohol is one of the most widely misused substances. Alcohol abuse affects not only the drinker but also the wider society. The World Health Organization (WHO) estimated approximately 2.5 million deaths per year due to the hazardous consumption of alcohol (World Health Organization, 2011; IAS, 2013). Regular excessive alcohol intake is known to be associated with an increased risk of many diseases such as liver cancer, infections, infertility, reduced immunity, esophageal cancer, depression and liver cirrhosis (Rehm et al., 2003). Early identification and treatment of alcohol use disorders (AUD) has the potential to reduce these risks. AUD is a condition of alcohol misuse in which the drinker drinks excessive amount of alcohol and has a continuous urge to consume alcohol. It has many symptoms such as progressive increase in alcohol intake to maintain a given level of intoxication, reduction in social activities, drinking search behaviour (searching for events or places which include alcohol drinking) and continuous drinking regardless of psychological, social and physical problems it produces (National Institute on Alcohol Abuse and Alcoholism, 2016). Current methods of AUD diagnosis include the use of clinical questionnaires such as the Alcohol Use Disorders Identification Test (AUDIT) and the Michigan Alcoholism Screening Test (MAST), but individuals with AUD are often reluctant to accept or disclose their AUD, making such questionnaires unreliable (Kroke et al., 2001). Some biomarkers such as gamma glutamyltransferase (GGT), mean corpuscular volume (MCV), alanine aminotransferase (ALT) andaspartate aminotransferase (AST) have also been used to diagnose AUD, but these biomarkers lack specificity and sensitivity (Adias, Egerton, & Erhabor, 2013; Kroke et al., 2001).GGT, ALT and AST could not discriminate between alcoholic and non-alcoholic liver diseases which impinge on their sensitivity and specificity in diagnosing AUD. MCV can be raised in vitamin B12 and folate deficiencies, non-alcoholic diseases and bone marrow disorders which limit its use in AUD diagnosis (Litten, Bradley, & Moss, 2010).

Metabolomics is the quantitative analysis of the metabolites in biological samples such as blood or urine (Corona, Rizzolio, Giordano, & Toffoli, 2012) in an aim to find novel biomarkers and to get better understanding of the pathways that lead to changes in the levels of

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metabolites due to disease, drugs or environmental factors (Harrigan, Maguire, & Boros, 2008). Some recent studies have used metabolomics to study alcohol consumption in animals and humans, but these studies focused only on investigating organ injury due to alcohol consumption, and did not explore the metabolic variations due to the chronic use of alcohol (Loftus et al., 2011; Jaremek et al., 2013; Gika et al., 2012).

In a previous paper, we reported the identification of AUD metabolic fingerprint in urine using ^{1}H NMR metabolomics analysis (Mostafa et al., 2016). In this study, we aimed to identify AUD metabolic fingerprint in plasma for the same subjects using ^{1}H NMR metabolomics analysis.

2. Methods

2.1. Recruitment of volunteers and sample collection

This study was conducted on three groups; individuals with AUD, social drinkers and alcohol-naive controls. The study was reviewed and approved by the medical research and ethics committee (MREC) of ministry of health of Malaysia. The recruitment of the volunteers was from June 2013 to November 2014. The volunteers of the three groups were between 18 and 60 years old, able to self-consent and had consented before participation in the study. The AUD patients were recruited from patients of the psychiatric department of Hospital Pulau Pinang (HPP) who had been diagnosed and assessed by the physicians at the respective department. The diagnosis was based on clinical AUD questionnaire and/or the NIH criteria (Zieve & David, 2011). All AUD volunteers were newly diagnosed AUD patients and none of them had started AUD treatment or had been on any AUD treatment before. The social drinkers and controls volunteers were recruited individually from the visitors and family members of patients in HPP. Each social drinker volunteer was assessed by the physician for being a non-AUD drinker based on the absence of NIH AUD criteria. All controls volunteers were alcohol-naïve who claimed never to have drunk alcohol before. In the three groups, volunteers were excluded from the study if they had been diagnosed with HIV or any blood transmitted disease, liver cirrhosis, or acute or chronic infectious diseases at the time of sampling. Further details on volunteers' recruitment were reported in our previous paper (Mostafa et al., 2016).

Blood samples (5 mL) were collected in EDTA tubes to prevent coagulation (Tuck et al., 2009).Samples were centrifuged at 12000 *rpm* for 10 min at 4 °C (MIKRO 22 R, Hettich Zentrifugen, Germany). The separated plasma was transferred into new Eppendorf tubes, and immediately stored at −80 °C.

2.2. Sample preparation for ^{1}H NMR analysis

The plasma samples were thawed at 4 °C on the day before the analysis. The Eppendorf tubes were then taken from the refrigerator and centrifuged at 12000 *rpm* for 5 min at 4 °C (MIKRO 22 R, Hettich Zentrifugen, Germany). For sample preparation, a 300 μL plasma sample was mixed with 300 μL of phosphate buffer and vortexed for few seconds. Briefly, the phosphate buffer was prepared by dissolving Na_2HPO_4, NaH_2PO_4, TSP and NaN_3 together then the final volume was completed with D_2O and ddH2O. The buffer was kept in the refrigerator at 4 °C. Then, 550 μL of the mixture was transferred into a 5 mm NMR tube (BRUKER, Switzerland). Further details on phosphate buffer preparation were elaborated in previous paper (Mostafa et al., 2016).

2.3. NMR spectroscopy

The samples were run in an NMR spectroscopy machine (BRUKER Ascend™ 500, Germany) with the sample temperature held at 300 K. The ^{1}H NMR analysis was processed using TopSpin 3.2 (BRUKER BioSpin, Germany). Based on the Bruker® protocol, the ^{1}H NMR experiment setups which were used to obtain the spectra in this study were 1D NOESY-presat (noesypr1d), CPMG-presat (cpmgpr1d) and *J*-resolved (Beckonert et al., 2007). Once the data obtained from TopSpin, it was imported to AMIX software to get an excel file of peaks intensities by normalized to total intensity and then was aligned to glucose peak at 5.22 ppm as a reference signal (glucose peak 5.22 ppm according to The Human Metabolome Database (HMDB)) as the TSP peak at 0.00 ppm was broad and not sharp (ROBERTS, SCHIRRA, LAVIN, & GARDINER, 2014). The resultant excel file was imported to Soft Independent Modelling of Class Analogies (SIMCA 13.0.3, Umetrics) software for statistical analysis and to identify the discriminating metabolites of AUD.

2.4. Statistical analysis

Multivariate principle component analysis (PCA) and orthogonal partial least squares-discriminated analysis (OPLS-DA) were performed on the ^{1}H NMR spectra and then the discriminant metabolites were further entered to univariate and multivariate logistic regression in order to develop a discrimination model between the AUD group, non-AUD social drinkers and controls. The significant metabolites were selected for biomarker identification using Bruker Biofluid Reference Compound Database (B-BIOREFCODE), Chenomx, HMDB and 2D Heteronuclear Single Quantum Coherence (2D HSQC) spectra. The inter-day reproducibility of these metabolites was tested using Intraclass Correlation Coefficient (ICC). For each patient, two plasma samples (main and duplicate) were run in ^{1}H NMR in two different days (around one month apart) to test the NMR machine stability and consistency.

3. Results

3.1. Demographic data

At the end of the data collection, the sample size of each group was as followed: 30 individuals with AUD, 54 social drinkers and 60 controls. The majority of the participants were Indians (47.2%) and male (68.7%).The mean age for the three groups was: AUD (45.7), social drinkers (39.46) and controls (37.13). The mean (SD) alcohol consumption of the AUD group was 16,363.83 mL (26,869.68) per week, while the mean (SD) alcohol consumption of the social drinkers group was 737.04 mL (1101.72) per week. Table 1 shows the demographics of the participants.

Table 1
Demographics.

Demographics	AUD n (%)	Social drinkers n (%)	Controls n (%)
1 – Gender[a,b]			
Male	30 (100)	41 (75.9)	28 (46.7)
Female	0 (0)	13 (24.1)	32 (53.3)
2 – Ethnicity[a,b]			
Malay	7 (23.3)	10 (18.5)	39 (65)
Chinese	0 (0)	17 (31.5)	2 (3.3)
Indian	23 (76.7)	26 (48.1)	19 (31.7)
Other	0 (0)	1 (1.9)	0 (0)
3 – Smoking[a,b]			
Smokers	27 (90)	31 (57.4)	10 (16.7)
Ex-smokers	0 (0)	5 (93)	2 (3.3)
Non-smokers	3 (10)	18 (33.3)	48 (80)
4 – Diabetes mellitus (DM)	1 (3.33)	3 (5.55)	2 (3.33)
5 – Hypertension (HTN)	3 (10)	3 (5.55)	2 (3.33)
6 – Age[c]			
Mean	45.7	39.46	37.13
SD	8.92	11.35	10.09

[a] p-value < 0.05.
[b] Chi-square test.
[c] Kruskal-Wallis test.