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Altered Metabolic Profile of Blood Plasma in Patients with Celiac disease: In-Vitro Proton Magnetic Resonance Spectroscopy Study

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Abstract Present study investigated the metabolic profile of blood plasma of patients with Celiac disease (CeD) and healthy controls (HC) using in-vitro nuclear magnetic resonance (NMR) spectroscopy to get an insight into the metabolic abnormalities in CeD and to determine the biomarker/s, if any, for the diagnosis of CeD. The concentration of metabolites was determined using one-dimensional NMR at 700 MHz. The blood plasma from patients with CeD showed significantly higher ($p<0.05$) concentrations of several metabolites such as alanine (Ala), glycine (Gly), acetate (Ace) and creatine (Cr) compared to the HC subjects. While, concentration of creatinine (Crn) was significantly lower ($p<0.05$) in blood plasma of CeD patients. These findings suggested that the gluten induced inflammation resulted in significant alterations in metabolic activity in CeD. Partial least square discriminant analysis (PLS-DA) clearly distinguished the two groups in separate clusters on the score plot of samples from CeD patients and HC. Our results provided an insight into the alterations in metabolic pathways in CeD and indicated that the NMR spectroscopy of blood plasma in a large cohort of CeD patients may aid in determining the non-invasive biomarker/s for the diagnosis of CeD.

Keywords: *Celiac disease, MR spectroscopy, metabolic profile, metabonomics, blood plasma*

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1. Introduction

Celiac disease (CeD) is a chronic gastrointestinal autoimmune disorder that specifically affects the small intestine. The dietary intake of gluten and related prolamines present in cereals like wheat, rye, and barley results in inflammation of small intestine that leads to villous atrophy in genetically susceptible individuals (HLA- DQ2 and HLA-DQ8) [1]. The disease presents with a wide range of clinical manifestations that include chronic diarrhoea, abdominal pain, weight loss, fatigue and anaemia. Besides, CeD is reported to be associated with a large number of extra-intestinal manifestations, such as bone fractures, infertility, neurological disorders, skin lesions and liver abnormalities [2,3] which contribute to the complexity of diagnosis of CeD.

The CeD is reported in both children and adults with a prevalence of approximately 1% in worldwide [4,5]. Both genetic and environmental factors play an important role in the pathogenesis of CeD, consequently affecting the metabolism. Thus, metabonomics study may provide valuable information on biomarkers that could be used for the diagnosis of CeD. Metabonomics study provides information on time related quantitative multiple metabolic changes that occur in response to environment, pathophysiological stimuli and genetic modulation by investigating the biological samples, such as blood plasma, urine, using analytical spectroscopic techniques like high-resolution nuclear magnetic resonance (NMR) spectroscopy combined with multivariate statistical methods [6].

Recently, a few studies have documented the metabonomics of blood plasma and urine in CeD patients using NMR spectroscopy and multivariate analysis methods [7,8,9,10]. Our group reported the characteristic metabolic signatures of villous atrophy in the intestinal mucosal biopsies of patients with CeD and compared with the data of disease control subjects using proton (^{1}H) NMR spectroscopy [10]. However, till date no study has determined the absolute concentration of metabolites in the blood plasma of CeD patients. The concentration of metabolites and their analysis combined with multivariate statistical modelling may present a comprehensive view of the alteration of metabolic pathway.

Therefore, the objectives of this study were (a) to determine the absolute concentration of metabolites in the blood plasma from patients with CeD and the healthy controls (HC) using ^{1}H NMR spectroscopy and (b)

analyze the data with multivariate analysis to determine biomarker/s which may be used for the diagnosis of CeD patients and (c) to obtain an insight into the altered CeD metabolism.

2. Patients and Methods

This study was carried out between August 2012 and November 2014. The Institute Ethics Committee approved the study and an informed consent was obtained from each participant.

2.1. Patients and Controls

Twenty treatment naïve patients with CeD (mean age 32.5±10.4 yrs; female 11; male 9) were recruited from the Celiac Disease Clinic of our Institution. The diagnosis was based on the positive serology and confirmed by histological examination of small bowel biopsies taken from the second part of the duodenum. Marsh-Oberhuber classification was applied for gradation of mucosal changes.

Twenty healthy subjects (mean age 27.5±3.4 yrs; female 8; male 12) serologically tested as negative for anti-tissue transglutaminase antibodies (tTGA), served as a control group (HC).

2.2. Sample Collection and Processing for NMR Spectroscopy

Blood samples were collected from each subject in the morning pre-prandial after overnight fasting. Peripheral venous blood samples (5 ml) were collected in a heparinized vacutainer and centrifuged at 2000 RCF for 10 minutes at 4°C. Then plasma was separated and stored at –80°C until NMR experiment were carried out. For NMR spectroscopy, deuterium oxide (D_2O), 0.5 mM of sodium trimethyl-silyl-[2, 2, 3, 3-H_4] propionate (TSP) and 0.5mM of sodium formate were added to the blood plasma samples. TSP served as a chemical shift reference while formate was used as concentration standard for the ^{1}H NMR studies.

2.3. NMR Spectroscopy

Proton NMR spectroscopy of the blood plasma samples was carried out on a narrow bore spectrometer operating at 700 MHz (Agilent, U.S.A.) using one dimensional (1D) Carr-Purcell-Meiboom-Gill (CPMG) sequence with presaturation. The typical parameters for 1D experiment were: spectral width of 9000 Hz; data points 32 K; number of scans 64; spin echo delay, τ of 15 ms and a relaxation delay of 70s. A long relaxation delay was used, since formate typically has a long T1 relaxation time. Two-dimensional (2D) total correlation spectroscopy (TOCSY) experiments were carried out for assignments of resonances.

The data was processed on a Dell 390N, PC, Red Hat Enterprise Linux workstation using the Varian software, Vnmrj 2.3 A. The free induction decays were multiplied by an exponentially decaying function prior to Fourier transformation. Spectra were manually phased using zero and first order corrections.

2.4. Quantification of Metabolites

The concentration of metabolites were determined by comparing the integrated intensity of isolated resonance of the compounds of interest with that of the intensity of the formate signal using the formula as presented earlier by us [11,12,13].

2.5. Statistical Analysis

The concentration values of metabolites were reported as mean ± standard deviation. Univariate analysis was carried out using SPSS software (SPSS version 11.5 for windows: SPSS, Inc., Chicago, IL). For comparison between the two groups, a Student's t-test/Wilcoxon Rank Sum test was used. A p-value <0.05 was considered as significant. Multivariate analysis was performed using Unscrambler 10.2 (Camo, Oslo, Norway). Principal component analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) were carried out on the concentration data of the metabolites. PCA is a powerful method of data extraction, which finds combinations of variables that describe trends in large data, called principal components and visualized in scores and loading plots. Score plots help in identifying clusters and outliers in the dataset where as loading plots show contributing variables to each principal component. PLS-DA shows the relationship between the spectra and the group information using multivariate regression methods. The metabolites responsible for the separation between the two groups are shown in loading plots.

Variables were mean centered and unit variance scaled and explored by PCA for initial analysis and detection of inherent trends and outlier detection. Further, a classification model was created from metabolites present in the blood plasma samples of patients with CeD and HC using PLS-DA.

3. Results

In all, 25 metabolites were assigned unambiguously in the blood plasma of patients with CeD using 1D and 2D TOCSY (spectrum not shown). The concentrations [(µM/L (mean ±SD)] of metabolites were calculated from the blood plasma of CeD patients and HC and is presented as histogram in Figure 1.

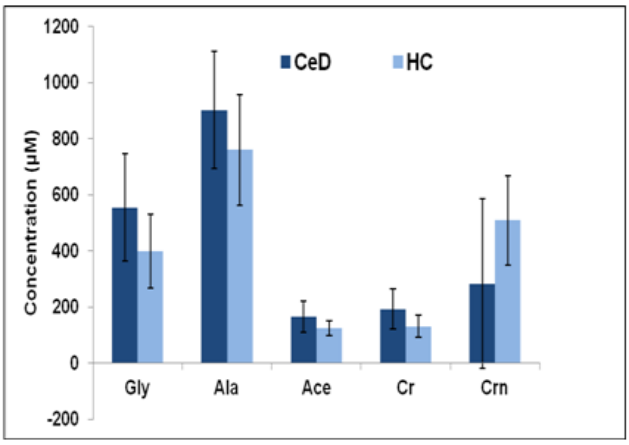

Figure 1. Histogram showing the comparison of concentration of metabolites between patients with CeD and HC

Univariate analysis showed significantly higher ($p<0.05$) concentrations of alanine (Ala), glycine (Gly), acetate (Ace), and creatine (Cr) in patients with CeD in comparison to HC subjects. Whereas, a significantly lower ($p<0.05$) concentration of creatinine (Crn) was observed in CeD patients as compared to the HC group.

Figure 2 shows a PLS-DA classification score plot that depicts the differentiation of the two groups; patients with CeD and the HC subjects in separate quadrants. The cross validated predicted fraction (Q2) value of the PLS-DA model for the blood plasma of CeD patients versus HC was 0.71 indicating the high reliability and strong predictive power of the PLS-DA model.

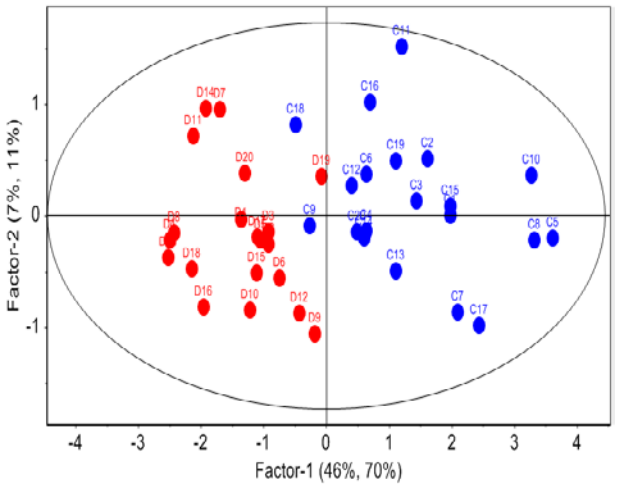

Figure 2. PLS-DA scores plot showing the separation between patients with CeD (D,●) and HC (C,●)

4. Discussion

In the present study, ^{1}H NMR-based metabonomics approach applied on the blood plasma samples demonstrated the altered metabolic activity of the patients with CeD compared to the HC subjects.

Our data showed significantly higher concentration of Ace in CeD patients compared to that seen in HC subjects. Ace is an end product of the lipid metabolism, and its elevation in blood plasma may reflect increased utilization of lipids in order to meet energy requirements in CeD patients than in HC. Earlier study has reported the higher levels of glucose (Glc) and β-hydroxybutyrate in blood sera of CeD patients [7] and suggested that the ketone bodies are being used as energy source in the patients with CeD instead of Glc which is in agreement with our data. Since, the energy generation through these metabolic pathways is not as efficient as through the oxidation of Glc, it may be the metabolic basis of fatigue, which is usually experienced by the patients with CeD [7].

Our data also revealed a significantly higher concentration of gluconeogenic amino acids, namely, Gly and Ala in the blood plasma of patients with CeD than that observed in HC subjects. This indicated that the amino acids, Ala and Gly probably might not have been utilized for gluconeogenesis in CeD. Further, the utilization of ketone bodies has been shown to inhibit gluconeogenesis pathway which supports our findings [14].

Furthermore, our data showed significantly decreased concentration of Crn in the blood plasma of CeD patients as compared to the HC subjects. The low Crn levels in CeD patients may most likely be due to malnutrition as a consequence of mal-absorption [15]. In patients with CeD, villous atrophy reduces the nutrients absorption, as a result, body needs an alternate energy source and it may utilize the fat and the protein present in the muscle tissue. This may probably leads to a significant loss of muscle mass and low Crn level observed in CeD patients [15]. Bertini et al. also reported significantly lower levels of amino acids (leucine, valine, proline, methionine) and Crn in blood plasma of CeD patients in comparison to controls [7]. Our results also revealed elevated levels of Cr in the blood plasma of CeD patients than healthy controls. Since, Cr is a prominent compound of metabolism which is involved in the energy supply for muscle metabolism, this imply that ATP production is altered in CeD patients which may lead to energy deficiency in CeD [16].

Further, multivariate analysis PLS-DA model clearly demonstrated significant biochemical dissimilarities in CeD patients and HC subjects and the samples from the two groups formed separate clusters on the score plot (Figure 2). The PLS-DA model was built with a significant predictive accuracy (Q2=0.71). The loading plot provided the semi-quantitative information and depicted that several metabolites like tyrosine, choline, Ace and Crn contributed as discriminating markers between CeD patients and HC subjects.

5. Conclusions

The data showed that quantitation of metabolites provides an insight into altered metabolic pathways in CeD patients and showed that several metabolite may play an important role as biomarker/s for diagnosis of CeD, however, the study needs to be carried out in a large cohort of patients at different sites.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

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