Metabonomics study of urine in patients with Celiac disease using in-vitro proton MR Spectroscopy

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Introduction: Celiac Disease (CeD) is an autoimmune enteropathy caused by ingestion of gluten and related prolamines present in cereals in genetically predisposed individuals[1]. CeD affects around 0.7-3.0% of the general population in both developed and developing countries and the prevalence is increasing over the years[2]. Currently, the screening of CeD is based on the serological markers such as endomysial (EMA) and tissue transglutaminase (tTG) IgA antibodies but the diagnosis is established by biopsy of the small intestine on endoscopy. However, the histopathological evaluation involves several problems. Firstly, it is an invasive procedure and secondly, sometimes the biopsy specimens are poorly oriented which may increase the risk of false positive or negative results[3]. Therefore there is a need of biomarker/s for villous abnormality which could be used for the diagnosis of CeD and help in the patient management. Thus the objectives of present study are: (a) to determine the concentration of metabolites in urine sample of patients with CeD and compare them with patients with gastro oesophagus reflex disease (GERD) and dyspepsia who serve as diseased controls (DC) and healthy controls (HC), using in-vitro NMR at 700 MHz, and (b) to understand the disease pattern using multivariate analysis and investigate the biomarker/s which would help in differentiation of CeD from controls.

Patients and Methods: Thirty patients with CeD (n=30; mean age 25.5±10.5 yrs) and fifteen HC (n=15; mean age: 28.9±5.5 yrs) were recruited for this study. Nineteen diseased (n=19; mean age: 33.5±9.6 yrs) controls were also included in this study. An informed consent was taken and the Institute Ethics Committee approved the study. All subjects were treated according to standard treatment regimen. The diagnosis of CeD was made on the basis of European Society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN). Urine samples were collected in morning pre-prandial and stored at -80°C until NMR spectroscopic analysis. For NMR spectroscopy, phosphate buffer was added to the urine sample to maintain the pH and sodium trimethyl silyl- (2,2,3,3-H4) propionate (TSP) was added to serve both as a chemical shift reference and concentration standard for the proton NMR . ^{1}H NMR spectra of urine samples were carried out at 700 MHz (Agilent, U.S.A.) spectrometer at 298K. Typical parameters for 1D were: spectral width=11,000 Hz; data points=32 K; number of scans=64 and relaxation delay=14 seconds. Quantification of metabolites was carried out by using the Chenomx NMR suite 7.5 software. Comparison of metabolites in celiac patients and controls were carried out using student's t- test. Probability values of 5% were considered significant ($p<0.05$). Partial least squares-discriminant analysis (PLS-DA) was performed to explore biochemical dissimilarities between patients using Unscrambler 10.2 (CAMO Software,Oslo, Norway).

Results: In all concentration of 45 metabolites were determined by using Chenomx NMR suite. The concentration of five metabolites that showed statistical difference with HC and DC are presented in Table 1. PLS-DA showed clear distinction among CeD patients, DC and HC (Figs.2 & 3). However no difference was observed between DC and HC.

Table 1: Concentration (mM/L) of metabolites in CeD patients, DC and HC.
(* denotes, $p<0.05$ between CeD & HC and # denotes, $p<0.05$ between CeD & DC)

Metabolites	CeD (n=30) Mean±SD	DC (n=19) Mean±SD	HC (n=15) Mean±SD
Citrate	1.27±1.23	1.73±1.56	2.59±1.81*
Creatinine	9.53±6.70	14.08±8.95#	14.85±9.48*
Fucose	0.43±0.31	0.35±0.17	0.25±0.10*
Phenylalanine	0.37±0.25	0.17±0.15#	0.19±0.11*
Trans-aconitate	0.30±0.17	0.12±0.07#	0.11±0.05*

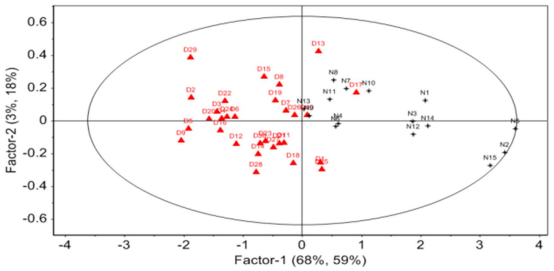

Fig2: PLS-DA plot for the CeD patient (red) & HC (black).

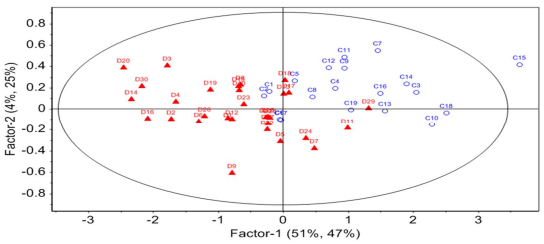

Fig3: PLS-DA plot for CeD patient (red) & DC (blue).

Discussion: Our results showed higher level of trans-aconitate and phenylalanine in the urine sample of CeD patients and lower level of creatinine as compared to healthy controls (HC) and diseased controls (DC). Trans-aconitate is a citric cycle intermediate which is the main metabolic pathway that provides energy to the body. Abnormal spilling of trans-aconitate may indicate mitochondrial inefficiencies in energy production. Higher concentration of phenylalanine in urine sample of CeD patients suggests protein malabsorption in CeD. Lower creatinine indicates lower muscle mass which is caused by a disease or due to the deficient protein diet. Thus the decreased creatinine level in urine sample of CeD patients is indicative of malabsorption in CeD. In addition, our results revealed higher concentration of fucose and decreased concentration of citrate in CeD patients as compared to HC. It is reported that fucosidase activity is increased in certain pathological conditions of liver which may result in increased urinary excretion of fucose[4]. Higher level of fucose in urine may be due to liver abnormalities associated with CeD. A significant decrease in the level of citrate may be due to the chronic diarrhoea. Chronic diarrhoea results in loss of base and decreased urinary citrate excretion due to impairment in the gastrointestinal absorption.

Conclusion: Our results provide an insight to understand the metabolic alterations occur in CeD. Significantly elevated levels of trans-aconitate and fucose were observed which may have the potential to serve as putative biomarker/s for differentiation of CeD from controls.

References: (1) Husby S et al. *J Pediatr Gastroenterol Nutr*, 2012; 54: 136–60; (2) Rubio-Tapia A et al. *Gastroenterology*, 2009; 137: 88–93; (3) Collin P et al. *Eur J Gastroenterol Hepatol*, 2005; 17: 85–91; (4) Jezequel-Cuer M et al. *Liver* 1992; 12: 140–146.