Evaluation of the Utility of Amino Acid Citrulline as a Surrogate Metabolomic Biomarker for the Diagnosis of Celiac Disease

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ABSTRACT

INTRODUCTION: Citrulline is regarded as a biomarker for celiac disease (CD). Its utility for assessment and evaluation of additive predictive value for latent, potential CD and first degree relatives (FDRs) needs exploration.

METHOD: Consecutive 558 index cases diagnosed as per European Society for Pediatric Gastroenterology and Nutrition (ESPGHAN) 2012 guidelines and their 1565 FDRs were evaluated over five and half year period. Serology negative FDRs at initial visit and follow ups were served as controls. HLA typing for DQ2 and DQ8 genotypes, along with plasma and dried blood spot (DBS) filter paper citrulline were evaluated.

RESULTS: Median plasma citrulline values were 20.1 and 37.33 µMol/l in cases and controls (P < .001). Cut off values for Marsh grade 3a, 3b, and 3c were 35.0, 32.8, 25.26 µMol/l in CD patients and 36.51, 30.10, 25.26 µMol/l in biopsy proven FDR. Increasing trends of plasma citrulline levels with decreasing tTG-IgA levels were observed on follow up. Low plasma citrulline levels were observed with HLA DQ 2.5 genotype (P < .05). Agreement between DBS and plasma citrulline was 94.8%.

CONCLUSION: Citrulline is a good surrogate biomarker for identification of histopathological grade of damage, extent of mucosal recovery and has negative correlation with tTG-IgA. It identifies the silent and latent phase of CD. DBS citrulline provides adequate information and can be used for monitoring CD patients at remote locations.

KEYWORDS: Celiac disease, surrogate metabolomic biomarker, plasma citrulline, enteropathy, DBS citrulline, gluten free diet, HLA DQ antigen

Introduction

Celiac disease (CD) is systemic, chronic, small intestine immune mediated enteropathy triggered by the ingestion of gluten from wheat, barley and rye in genetically susceptible individuals.[1] The prolamin fraction of gluten is probably antigenic due to the presence of glutamine and proline rich amino acids residue in its primary structure.[2,3] The course involves an inflammatory immune response that damages the mucosal lining of the small intestine resulting in the malabsorption of micro and macronutrients.[4-6] Almost 1.4% peoples are affected with CD and life-long gluten free diet (GFD) is the only treatment available that benefits patients with CD.[7,8]

Serological tests play an integral role in the diagnosis of CD while the search is on for a better biomarker. The major serological markers in clinical use are anti-tissue transglutaminase antibody type 2 (tTG-IgA), anti endomysial antibody (EMA) and antibody against deamidated forms of gliadin peptides (DGP-IgA).[9] These markers being IgA subtypes may miss the diagnosis in concomitant IgA deficiency, which is reported 10 to 15 times higher in CD.[10] Because of the pitfalls of serological markers, histomorphology of intestinal biopsy assessed by Marsh-Oberhuber grade 2 and above still remains the gold standard.[1,11] Upper GI endoscopy is a reasonably safe procedure but is invasive, requires patient preparation, has sedation related complication risks, requires procedural modifications for pediatric, elderly age group and high-risk populations and is discomforting to the patient. It is not uncommon for the patients and the caregivers to not report on their appointment dates and denial of consent

despite extensive counseling. It is also very difficult to diagnose cases with Marsh grade 1 mucosal lesion as this condition does not always correspond with CD and has a wider differential diagnosis. Mostly, cases with Marsh grade 1 are doubtful and uncertain and this is a potential gap with no response. Thus, there is an impending need of a non-invasive marker that can screen individuals at risk for CD, distinguish between the varied presentations, correlate with the severity of the intestinal lesions and extent of mucosal damage, monitor the histological response to GFD and correlate with the symptom type.

Citrulline is a non-essential amino acid (Aa) and an intermediate of the urea cycle for which no genetic code is available. Circulating citrulline is specifically produced by proximal small bowel gut enterocytes, from the middle and upper part of intestinal villi.[12] Citrulline has been proposed as a potential marker of intestinal function as exogenous diet cannot replenish citrulline. Citrulline has a utility for the decision about post-surgical permanent intestinal function and correlates with residual bowel length.[13] Citrulline levels are informative about villous atrophy associated with intestinal disease, small intestine transplantation, necrotizing enterocolitis in new born, small bowel resections and chemotherapy induced mucosal enteropathy.[14] Though its utility in CD had been evaluated by some authors, its value in first degree relatives (FDRs) and additive value over HLA genotype is still unexplored. Its correlation with degree of intestinal lesion at presentation and monitoring in this subset may yield important information and may possibly fill the gap for the diagnosis of CD in Marsh grade 1 mucosal lesion.

Based on these qualities of amino acid citrulline, the aim of this study was to evaluate plasma citrulline level in children with CD and in their FDRs, correlate these with the degree of intestinal lesions at the time of diagnosis and monitor the trend of citrulline levels after the initiation of GFD. It was also envisaged to enhance detection of potential CD over a specified time period of 18 months. This has been scarcely attended to in published literature

Materials and Methods

Study population

This was a prospective longitudinal cohort study conducted at Genetic laboratory, Department of Pediatrics, Division of Genetics, Maulana Azad Medical College and Associated Lok Nayak hospital, a tertiary care hospital, in North India in between February 2014 to July 2019. Study was approved by institutional ethical committee as per Indian Council Medical Research guidelines (ICMR), vide letter no- F.1/IEC/MAMC/(31)/3/2012/No:236. Written informed consent from the parents of all study subjects were obtained. All the patients (1-18 years) with a clinical suspicion of CD, visiting outpatient department (Gastroenterology and Genetics clinic) of our hospital as well as already diagnosed case with confirmatory biopsy were included. Thus, referrals from other institute/hospital with a confirmed diagnosis were also recruited in the study. Patients of CD and their FDRs with

chronic kidney disorders (CKD) and citrullinemia as an inborn error of metabolism were excluded from the study.[15,16] Exclusion criteria also included known patients with HIV, acute rejection after intestinal transplantation, short bowel or inflammatory bowel syndrome, patients on chemotherapy. All the FDRs without known kidney disease were enrolled. Diagnosis of CD was made as per the guidelines proposed by European Society of Pediatric Gastroenterology and Nutrition (ESPGHAN) in 2012.[17] All the cases accompanied by FDRs were requested to get other FDRs for serology screening (tTG-IgA). HLA typing for DQ2 and DQ8 genotypes were performed in both index cases and FDRs. Asymptomatic and serology negative FDRs were subsequently used as controls in the study. Scheduled follow up visits at 6 monthly intervals were mandated for both the cases and controls and after the institution of GFD for cases. tTG-IgA and citrulline estimation along with anthropometry, nutritionist review for compliance with GFD and a repeat dietary counseling was done.

Sample collection and storage

Total of 4 ml blood was collected out of which 1 ml blood for serum anti tTG-IgA assay and 3 ml blood for plasma citrulline assay by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) and HLA testing for DQ2 and DQ8 genotypes using PCR-SSP (Polymerase Chain Reaction-Sequence Specific primer) was used. About 1 ml plasma was transferred into pre-treated sodium salicylic acid (SSA) vials, mixed vigorously until it showed a milky froth appearance and kept under –20°C for until analysis. SSA vials were prepared by dissolving 200 gm of SSA (Merk, Darmstadt, Germany) in 1 l of absolute ethanol, aliquoted in 1.5 ml micro centrifuge tubes and dried at 45°C to 55°C overnight.[18] For dried blood spot (DBS) citrulline analysis by Tandem Mass Spectroscopy (TMS), 100 μl of whole blood on 903 S&S GE Whatman filter paper (DBS) were used, air dried and kept at 4°C till analysis. DNA extraction from 1 ml EDTA blood was performed by phenol chloroform method with minor modifications and stored in –20°C until analysis.[19]

Laboratory investigations

Anti-tTG-IgA antibody testing. The method employed for the detection of anti tTG-IgA antibody was quantitative Enzyme Linked Immunosorbent Assay (ELISA). Commercially available ELSA kits from HYCOR Biomedical (CA, USA) specific for IgA antibodies against tissue Transglutaminase (tTG) were used. Assay plate was read on Microplate reader, Model 680, BIORAD (CA, USA). Results were interpreted as negative for <7 U/ml and positive for >7 U/ml as per the cut off assigned by kit manufacturer.

Intestinal biopsy and histopathology. All patients with elevated tTG-IgA titer (>7 U/ml), were advised to undergo upper

gastrointestinal (GI) endoscopy and collection of at least 4 punch biopsy specimens from duodenum, after informed consent. Newly diagnosed cases with positive serology and histopathological features of villus atrophy that is modified Marsh grade 2 or 3 were labeled as index cases.[17] FDRs of these index cases were screened with serology alone and those with positive results underwent endoscopy and biopsy procedures with a similar approach. Upper GI endoscopies and directed biopsies of patients till the age of 12 years were performed in the department of Pediatrics, Maulana Azad Medical College and associated Lok Nayak Hospital while the patients, above 12 years of age, endoscopic procedures were performed in the Department of Gastroenterology, Govind Ballabh Pant Institute of Post Graduate Medical Education and Research (GIPMER). Endoscopic features of scalloping, nodularity and mosaic pattern of the duodenal folds were noted. Histopathology analyses were performed in the Department of Pathology, GIPMER and the findings were graded using the modified Marsh classification.[20] Subjects classified with grade 2 and 3 were diagnosed as being affected by CD.

HLA genotyping. HLA typing for DQ2 and HQ8 genotypes was performed using rapid polymerase chain reaction, sequence specific primer (PCR-SSP) method. Primer sequences for HLA DQA1*0501, DQB1*0201, DQA1*0201, DQB1*0202, DQA1*0301 and DQB1*0301 were taken from literature.[21-23] Reactions were performed in 25 μl of volume with minor modification in the protocol described in the literature and run over Veriti (Applied Biosystems, CA, USA) thermal cycler. Amplified products were run over 3% agarose gel electrophoresis and visualized on Gel Doc EZ system using Image Lab software, version 5.0 (BioRad, CA, USA).

Plasma citrulline assay. Citrulline estimation was done using Reverse Phase HPLC method, ECLIPSE AAA Column (5 μM, 4.6 × 150 mm) with pre column derivatization using OPA (Ortho Pthalaldehyde) and FMOC (9-flueroenylmethyl chloroformate) on Agilent 1200 series. Standards, calibrators and samples run were 12 minutes long with a post run time of 5 minutes. The pump was set to give a flow of 1 mL/minutes of buffer A (40 mMol N_a2HPO_4, pH 7.8) during the sample run and buffer B (Acetonitrile (45): methanol (45): water (10)) during the post run period. Calibration curve was made with 6-point calibrators and the curve was linear to 3.125, 6.25, 12.5, 25, 50, and 100 μmol/L. Identification of peaks was performed using fluorescence detector, excitation at 340 nm and emission at 450 nm with photomultiplier tube (PMT) gain being 11. Peak analysis was done using Agilent Chemstation 2.0 software. Standard for citrulline were procured from Sigma-Aldrich, Missouri, USA and other reagents were from Agilent Technologies (CA, USA).

Dried blood spot filter paper citrulline assay. Dried blood spot (DBS) filter paper citrulline analysis was performed on 3200 MD Qtrap, TMS (Applied Biosystems, CA, USA) equipped with Flexor (Perkin Elmer, Turku, Finland), liquid chromatography auto sampler. Blood spots of negative and positive controls with patient's samples were punched using Panthera (Perkin Elmer, Turku, Finland) automated puncture. Sample extraction, internal standard reconstitution, incubation and run was performed as on Neo-Base, Non-Derivatized MS-MS kit (Perkin Elmer, Turku, Finland). Results were analyzed using Analyst Program Launcher software (Quantity One 1.4.1 version).

Management and follow up. Once CD was confirmed, counseling about the need for GFD and micronutrient supplementation was imparted. The monitoring for compliance was on a scheduled specialty day with other patients with similar diagnosis and counseling about the vital importance of follow up. Those not reporting were recalled telephonically twice for ensuring compliance. Micronutrient supplementation was done on outpatient basis, in concordance with the treating team.[24]

Statistical analysis

Statistical analysis was performed by using R software, version 3.6.2. Qualitative data were depicted as number (percentages) and the differences among the groups were computed by Fisher exact test and Chi square test. Quantitative data were presented as median (Interquartile range, first and third quartiles) and the difference of medians among the groups were computed by Mann-Whitney *U* test. The *P*-value <.05 was considered to be statistically significant. Distribution of data was checked using Shapiro -Wilk test. Since most of the data followed a nonparametric distribution, Wilcoxon rank test was used to calculate the pairwise mean differences in citrulline and tTG levels across biopsy status groups. Cut point for R software was used to calculate area under curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV). CutpointR was used to generate optimal cutpoint/threshold values. For each value of threshold true positive, true negative, false positive, and false negative values were calculated. Using aforesaid mentioned matrices, we calculated the sensitivity and specificity which was used to calculate the area under curve using trapezoidal rule. Odd's ratio and Receiver operating characteristics (ROC) curve analysis were also performed to find out the optimal cut offs for different groups. The optimal cut off point was selected by choosing the threshold that had the maximum sum of sensitivity and specificity. Agreement analysis between plasma and DBS citrulline was performed using SPSS version 23. Interclass correlation coefficient was evaluated for the test of correlation. Paired *t*-test was performed for the evaluation of systemic differences. Bland-Altman plot was employed to compare the 2 different methods used for citrulline analysis.

Results

A total of 558 consecutively presenting index cases were evaluated. Of these, 6 had Marsh grade 2, 63 had grade 3a, 126 had grade 3b and 337 had grade 3c mucosal lesions on histopathology. Six patients who did not report post biopsy, 5 with inconclusive biopsy report and 15 with normal histopathology were excluded. Hence, 532 index cases were finally identified. A total of 1565 FDRs were screened by serum tTG-IgA. Fifty-eight cases did not return after initial screening, 62 cases did not give consent for biopsy and 25 cases developed CD during 24 months follow-up. Of the remaining 1420 FDRs, 263 (18.52%) were positive on initial serology and were advised to undergo upper GI endoscopy and biopsy. Twenty-five cases did not consent for biopsy, 5 underwent biopsy elsewhere and 6 with technical failure were excluded. Of the final 227 (15.98%) FDRs, 2 had grade 2, 99 had grade 3a, 43 had grade 3b, 82 had grade 3c. One case with non-conclusive histopathology was excluded from the analysis. Asymptomatic, tTG-IgA negative 1157 FDRs were recruited as controls.

For the purpose of analysis, we categorized our subjects into 3 groups, group 1 was constituted of both the index cases and serology positive FDRs which accounted for 758 collective cases (532 index cases and 226 biopsy proven FDRs). Group 2 consisted of 532 index cases, and group 3 consisted of 226 biopsy proven FDRs.

Baseline plasma citrulline and tTG-IgA levels at different Marsh grades

Median plasma citrulline levels in group 1 at baseline was 20.1 μMol/l (13.35-29.15 μMol/l) and in serum tTG-IgA negative FDRs (control group), the median level was 37.33 μMol/l (29.8-42.6 μMol/l), the difference being statistically significant ($P < .001$). Median plasma citrulline and serum tTG-IgA levels at Marsh grade 2 were 31.9 μMol/l (29.27-39.20 μMol/l) and 34.75 U/ml (11.83-189.5 U/ml), ($P = .137$, $r = -.573$). Median plasma citrulline and serum tTG-IgA levels in cases with grade 3a were 37.47 μMol/l (33.07-71.44 μMol/l) and 18.86 U/ml (9.84-31.62 U/ml), ($P = .029$, $r = .167$). Similarly, the levels in patients at grade 3b were 24.16 μMol/l (19.84-28.52 μMol/l) and 64.28 U/ml (48.75-74.38 U/ml), ($P < .001$, $r = -.346$) and grade 3c were 14.54 μMol/l (9.84-19.54 μMol/l) and 142 U/ml (107.45-175 U/ml), ($P = .003$, $r = -.143$) respectively.

Figure 1a to c depicts the scatterplot differentiating plasma citrulline and serum tTG-IgA in cases with Marsh 3a, 3b and 3c grade lesions.

Table 1 depicts plasma citrulline and serum tTG-IgA levels in group 2 and 3. Similar assessments were performed in 5 cases with inconclusive biopsy and median plasma citrulline and serum tTG-IgA levels in them were 12.26 μMol/l (9.84-19.84 μMol/l) and 23.7 U/ml (10.59 23.7-153.0 23.7 U/ml) ($P = .551$, $r = -.360$) and 15 with normal histopathology but EMA and HLA positivity, were 23.25 μMol/l (18.42-32.42 μMol/l) and 60.06 U/ml (23.0-145.43 U/ml) ($P = .844$, $r = .055$) respectively. These

findings indicate that plasma citrulline could successfully predict the potential CD cases (as the levels were less than the cut off value at Marsh 3c grade, <25.262 μMol/l) with high accuracy.

Receiver operating characteristic curve analysis

ROC analysis for citrulline as a diagnostic marker for gut enterocyte mass evaluation was performed. The cut off values for plasma citrulline in group 1 at Marsh grade 3a, 3b, and 3c were 35.0, 32.8, and 25.26 μMol/l. The cut off values at Marsh grade 3a, 3b, and 3c in group 2 were 36.51, 30.10, and 25.26 μMol/l and in group 3 were 39.40, 32.30, and 24.26 μMol/l respectively. Table 2 depicts the diagnostic accuracy of plasma citrulline in group 1, 2, and 3.

Figure 2a to c depicts ROC curves for cut off values evaluations in group 1.

Follow up analysis at baseline and at 6, 12, and 18 months after the institution of GFD

At March 3a grade mucosal lesions. Median plasma citrulline and serum tTG-IgA levels in cases with group 1 with Marsh 3a grade lesions at baseline and at 6, 12, and 18 months after the introduction of GFD were 37.47 μMol/l (33.07-41.44 μMol/l) versus 16.86 U/ml (9.86-31.43 U/ml), 41.26 μMol/l (35.58-46.57 μMol/l) versus 35.69 U/ml (22.75-57.59 U/ml), 39.85 μMol/l (33.26-46.23 μMol/l) versus 20.18 U/ml (6.49-33.69 U/ml) and 45.26 μMol/l (36.26-51.26 μMol/l) versus 6.58 U/ml (3.68-11.56 U/ml) respectively.

At March 3b grade mucosal lesions. In patients with Marsh 3b grade, plasma citrulline and serum tTG-IgA levels were 24.16 μMol/l (19.84-28.52 μMol/l) versus 64.28 U/ml (48.75-74.38 U/ml), 28.50 μMol/l (22.68-35.61 μMol/l) versus 49.74 U/ml (30.15-80.19 U/ml), 33.26 μMol/l (29.84-37.62 μMol/l) versus 22.56 U/ml (11.10-42.54 U/ml) and 40.15 μMol/l (36.84-46.58 μMol/l) versus 15.97 U/ml (7.26-35.26 U/ml) respectively.

At March 3c grade mucosal lesions. In patients with Marsh 3c grade, plasma citrulline and serum tTG-IgA levels were 14.54 μMol/l (9.84-19.54 μMol/l) versus 142 U/ml (107.45-172.83 U/ml), 21.65 μMol/l (15.84-29.56 μMol/l) versus 100.01 U/ml (66.38-129.42 U/ml), 30.26 μMol/l (23.69-34.84 μMol/l) versus 52.36 U/ml (26.54-80.28 U/ml) and 36.39 μMol/l (30.26-40.26 μMol/l) versus 20.0 U/ml (6.66-42.62 U/ml) respectively.

An inverse correlation was observed between the levels of plasma citrulline and serum tTG-IgA assays at follow up. Table 3 depicts the plasma citrulline and serum tTG-IgA levels at baseline and 6 monthly follow up. About 1146 (80.70%) of cases from FDRs were available for the first 6 months follow up, 1049 (73.87%) at 12 months, 924 cases (65.07%) at 18 months and 699 (49.22%) at 24 months. Twenty five (1.76%)
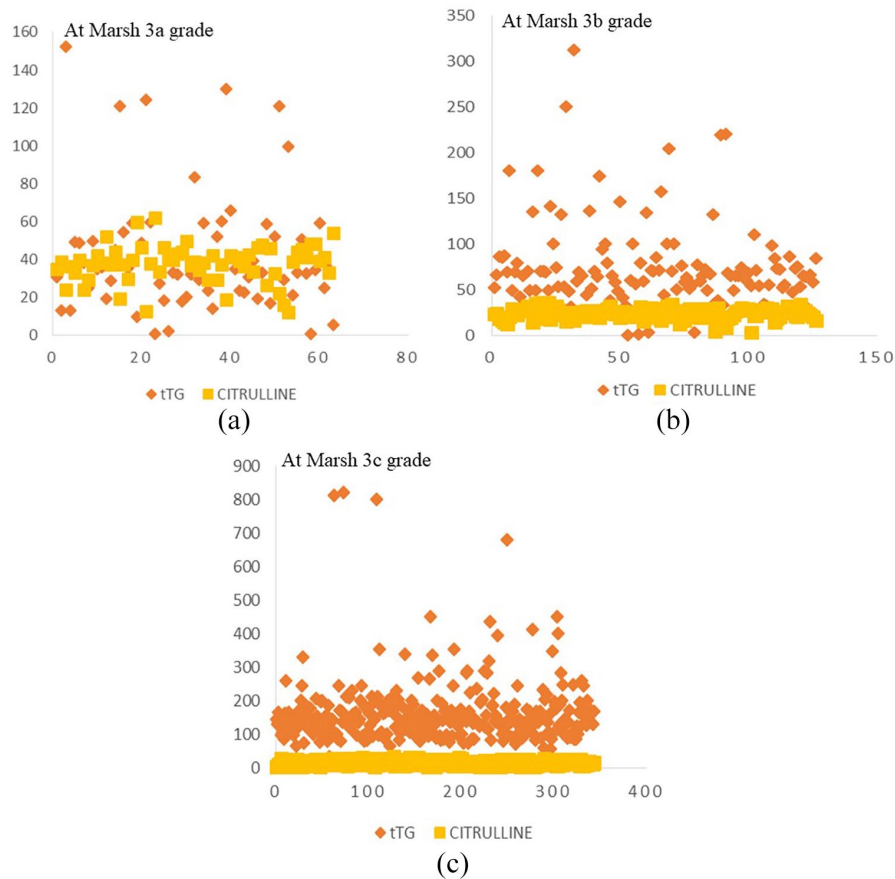
(a)

(b)

(c)

Figure 1. (a) Depicts the overlapping values of baseline plasma citrulline and serum tTG-IgA levels in patients with Marsh grade 3a, (b), and (c) depicts significant difference between plasma citrulline and serum tTG-IgA levels in patients with Marsh grade 3b and 3c suggesting that citrulline is a good predictor of damaged villous architecture in higher Marsh grade mucosal lesions.

Table 1. Plasma citrulline and serum tTG-IgA levels in group 2 and 3.

MARSH GRADE	MARKERS	GROUP 2	GROUP 3
3a	Plasma citrulline*	38.49 (33.13-42.36)	36.26 (31.42-39.26)
	Serum tTG-IgA#	35.0 (25.05-53.15)	11.15 (8.92-18.78)
3b	Plasma citrulline	24.5 (19.88-28.0)	23.25 (19.64-29.30)
	Serum tTG-IgA	65.66 (51.65-82.92)	52.75 (45.46-67.21)
3c	Plasma citrulline	14.38 (9.84-19.51)	15.77 (10.19-20.54)
	Serum tTG-IgA	143.48 (107.73-177.20)	133.26 (107.76-165.50)

Levels are in Median (IQR, Q1-Q3).
*Plasma citrulline (µMol/l).
#tTG-IgA (U/ml).

FDRs who were initially serology negative, turned positive over a period of 24 months that is 9, 9, 5, and 2 cases at 6, 12, 18, and 24 months respectively. Marsh grading on histopathological reporting could be done in 14 (0.98%) of these FDRs. Median (IQR, Q1-Q3) values of serum tTG-IgA and plasma citrulline levels of these cases at 6, 12, and 18 months were 11.59 U/ml (10.40-62.78 U/ml) versus 29.47 µMol/l (26.27-30.52 µMol/l), 27.9 U/ml (24.65-66.34 U/ml) versus 27.23 µMol/l (15.89-29.66 µMol/l) and 19.29 U/ml (14.36-26.65 U/ml) versus 26.43 µMol/l (20.34-31.42 µMol/l).

Figure 3 depicts the time related changes in plasma citrulline and serum tTG-IgA levels from baseline to 18 months

Comparison of plasma citrulline with HLA genotypes

For the comparison of plasma citrulline with HLA genotype study, subjects were further categorized into 4 groups. Group A constituted of 1859 total study subjects, group B of 532 index cases, group C of 226 biopsy positive FDRs and group D of 1101 controls with respect to their HLA genotype status.

Table 2. Diagnostic accuracy of plasma citrulline among group 1, 2, and 3.

VARIABLES*	GROUP 1			GROUP 2			GROUP 3		
MARSH GRADE	3A	3B	3C	3A	3B	3C	3A	3B	3C
AUC (%)	0.51	0.87	0.96	0.46	0.87	0.96	0.54	0.85	0.94
Sensitivity (%)	75.59	70.12	89.15	66.66	75.08	89.15	39.33	71.39	91.69
Specificity (%)	30.56	94.08	93.39	48.32	90.40	95.02	78.09	90.90	86.58
NPV (%)	86.60	38.03	80.81	95.22	34.34	77.56	13.48	13.88	49.65
PPV (%)	17.42	98.39	96.50	8.57	98.18	97.84	93.68	99.35	98.63
LR+	1.088	11.85	13.50	1.29	7.82	17.93	1.79	7.85	6.83
Odds ratio	1.36	37.32	116.30	1.87	28.38	157.21	2.31	24.95	71.26

*AUC, area under curve; NPV, negative predictive value; PPV, positive predictive value; LR, likelihood ratio.
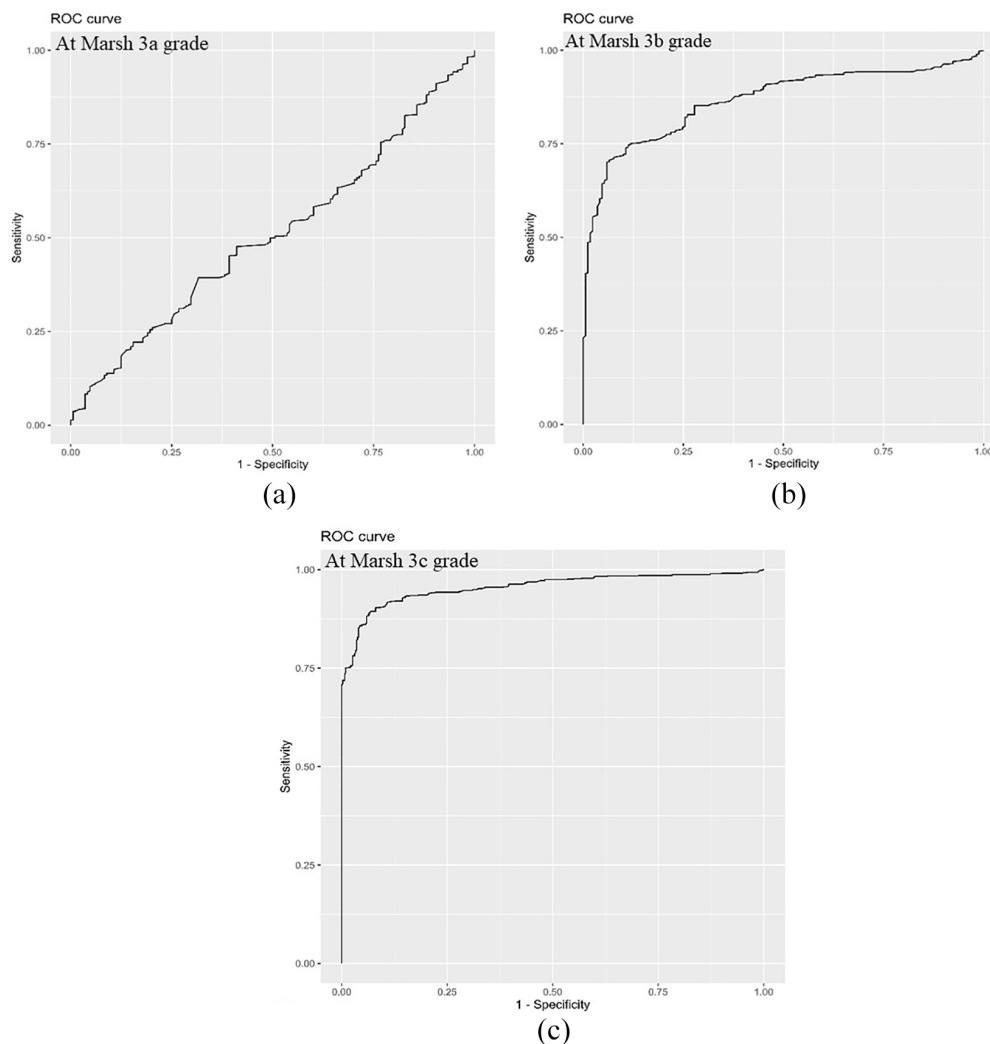

(a)

(b)

(c)

Figure 2. (a) to (c) Depicts the ROC curve for the evaluation of cut off values for plasma citrulline in Index cases and in serology positive FDRs at Marsh grade 3a (35.0 μMol/l), 3b (32.8 μMol/l) and 3c (25.26 μMol/l) mucosal lesions.

The median (IQR) plasma citrulline levels in HLA DQ2 and HLA DQ8 positive and negative cases in group A were 30.49 μMol/l (2.65-84.16 μMol/l) versus 36.26 μMol/l (2.39-84.59 μMol/l) (P < .001) and 25.52 μMol/l (4.26-54.51 μMol/l) versus 32.5 μMol/l (2.39-84.59 μMol/l) (P < .001) respectively.

In group B the levels were 18.48 μMol/l (3.07-62.1 μMol/l) versus 15.26 μMol/l (4.58-41.0 μMol/l) (P = .623) and 17.49 μMol/l (4.58-48.5 μMol/l) versus 18.51 μMol/l (3.07-62.1 μMol/l) (P = .376) respectively. The levels in group C were 27.16 μMol/l (5.26-68.26 μMol/l) versus 34.26 μMol/l (7.59

Table 3. Follow up levels of plasma citrulline and serum tTG-IgA levels in group 1 and 2.

	MARSH GRADE	6 MONTHS	12 MONTHS	18 MONTHS
Group 1				
	3a			
	Plasma citrulline*	41.70 (33.26-50.68)	38.15 (30.91-45.60)	45.52 (33.93-52.58)
	Serum tTG-IgA#	57.61 (30.23-82.51)	25.01 (13.98-40.86)	8.06 (5.26-14.33)
	3b			
	Plasma citrulline	27.18 (22.60-32.41)	32.95 (29.82-36.77)	40.15 (37.85-46.54)
	Serum tTG-IgA	56.05 (30.72-87.09)	23.25 (13.17-42.65)	16.25 (7.41-39.54)
	3c			
	Plasma citrulline	21.26 (15.84-28.95)	30.15 (22.65-34.03)	34.59 (30.23-40.17)
	Serum tTG-IgA	99.68 (65.91-128.87)	51.32 (25.89-79.15)	19.53 (6.58-41.26)
Group 2				
	3a			
	Plasma citrulline	41.26 (36.54-45.77)	39.75 (33.83-45.77)	45.26 (39.53-50.51)
	Serum tTG-IgA	26.51 (12.52-47.62)	15.62 (5.66-35.69)	6.11 (3.47-9.79)
	3b			
	Plasma citrulline	29.24 (27.57-34.10)	36.48 (32.59-39.54)	41.77 (37.09-46.59)
	Serum tTG-IgA	39.48 (28.90-52.56)	18.96 (10.23-40.32)	22.51 (10.39-34.43)
	3c			
	Plasma citrulline	22.36 (16.33-29.84)	32.40 (28.94-39.39)	39.48 (33.26-42.25)
	Serum tTG-IgA	111.29 (69.51-134.5)	59.88 (31.22-81.54)	29.62 (14.82-53.59)

Levels in Median (IQR, Q1-Q3).
*Plasma citrulline levels (μMol/l).
#Serum tTG-IgA levels.

-59.6 μMol/l) (P = .065) and 24.25 μMol/l (9.64-39.36 μMol/l) versus 28.13 μMol/l (5.26-68.26 μMol/l) (P = .758) respectively. Similarly, the levels in group D were 37.5 μMol/l (2.65-84.16 μMol/l) versus 36.55 μMol/l (2.39-84.59 μMol/l) (P = .610) and 37.58 μMol/l (4.26-54.51 μMol/l) versus 37.4 μMol/L (2.39-84.59 μMol/l) (P = .721) respectively. Table 4 depicts the detailed comparison analysis of plasma citrulline levels with the presence and absence of HLA genotype

Agreement between plasma citrulline and DBS filter paper citrulline assay

Correlation between plasma and DBS filter paper citrulline levels was evaluated in 405 cases and the obtained correlation was statistically highly significant (Interclass Correlation Coefficient = .50, P < .001). Systematic difference analysis depicted that the Mean ± SD of plasma citrulline was 20 ± 10 μMol/l and that of DBS citrulline was 19 ± 7.3 μMol/l, the difference being statistically highly significant (t = 4.159, P < .001). There was a 94.8% agreement between the 2 measures, that is, 94.8% of the observations had a difference, which was within the limits of agreement (±17.43). Figure 4 depicts the correlation and comparison of the means between plasma and DBS citrulline levels.

Discussion

We evaluated citrulline as a surrogate biomarker for CD in terms of assessment of disease burden, efficacy of treatment and additionally evaluated its predictive value in FDRs. We also evaluated the agreement between plasma citrulline measured by HPLC and as citrulline estimated by LCMSMS on DBS. There are limited studies that evaluated the role of citrulline in FDRs over a period of 2 years and its utility in the diagnosis of potential CD is scarcely available in published literature.

Description of plasma citrulline evaluation as a marker to assess the gut enterocyte mass reduction in patients with small bowel disease and in diarrheal or malnourished patients was given by Crenn et al.[25] Papadia et al[26] also reported citrulline to be a reliable marker of small bowel absorptive capacity with a strong correlation with small bowel length. These studies did

Table 4. Analysis of plasma citrulline levels with the presence and absence of HLA genotype.

HLA GENOTYPE	GROUP A (N = 1859)	GROUP B (N = 532)	GROUP C (N = 226)	GROUP D (N = 1101)
Median (IQR, Q1-Q3) plasma citrulline levels (μMol/L)				
DQA1*0501				
Presence	29.6 (2.65-84.16)	18.25 (3.07-62.1)	27.16 (5.26-68.26)	36.63 (2.65-84.16)
Absence	35.05 (2.39-84.59)	20.25 (4.1-56.2)	29.26 (7.59-59.69)	38.14 (2.39-84.59)
P-value	<.001	.034	.222	.800
DQB1*0201				
Presence	29.54 (2.65-84.16)	18.87 (3.07-62.1)	25.75 (5.26-68.26)	37.33 (2.65-84.16)
Absence	35.26 (2.39-84.59)	15.26 (3.52-45.26)	29.53 (6.26-59.6)	37.5 (2.39-84.59)
P-value	<.001	.003	.078	.856
DQA1*0201				
Presence	33.25 (3.26-76.25)	18.42 (4.1-46.5)	29.77 (5.26-59.69)	36.63 (3.61-76.25)
Absence	31.26 (2.39-84.59)	18.5 (3.07-62.1)	26.61 (5.49-68.26)	37.5 (2.39-84.59)
P-value	.485	.392	.218	.548
DQB1*0202				
Presence	28.5 (2.65-74.5)	17.51 (4.59-48.5)	22.36 (8.49-68.26)	37.5 (2.65-74.5)
Absence	32.3 (2.39-84.59)	18.5 (3.07-62.1)	28.26 (5.26-61.35)	37.5 (2.39-8.59)
P-value	.041	.853	.830	.736
DQA1*0301				
Presence	26.35 (4.26-54.51)	17.99 (4.58-48.5)	26.24 (18.48-28.28)	37.59 (4.26-54.51)
Absence	32.5 (2.39-84.59)	18.49 (3.07-62.1)	28.0 (5.26-68.26)	37.4 (2.39-84.59)
P-value	.002	.511	.953	.678
DQB1*0302				
Presence	20.26 (7.15-35.26)	14.59 (7.15-33.26)	26.24 (18.48-28.26)	35.26 (35.26-35.26)
Absence	36.26 (2.39-84.59)	18.48 (3.07-62.1)	28.0 (5.26-68.26)	37.5 (2.39-84.59)
P-value	.003	.438	.625	.769
DQA1*0501/DQB1*0201				
Presence	30.25 (2.65-84.16)	18.5 (3.07-62.1)	26.9 (5.26-68.26)	36.65 (2.65-84.16)
Absence	36.5 (2.39-84.59)	14.93 (4.1-41.0)	33.76 (7.59-59.6)	38.4 (2.39-84.59)
P-value	<.001	.161	.069	.799
DQA1*0201/DQB1*0202				
Presence	32.35 (2.65-76.25)	17.89 (4.1-48.5)	29.77 (5.26-68.26)	36.65 (2.65-76.25)
Absence	31.8 (2.39-84.59)	18.8 (3.07-62.1)	26.61 (5.49-61.35)	37.46 (2.39-84.59)
P-value	.688	.325	.216	.616

point evaluation rather than a longitudinal follow up or a single follow up. Its predictive value in FDRs over 24 months patients was evaluated in our study. Plasma citrulline levels also seemed to be a good biomarker in CD with an ability to distinguish between symptomatic subjects and serology negative healthy FDRs at presentation. Our study also entailed a follow up for 24 months in those negative at presentation. This is probably the first study to evaluate the plasma citrulline levels in FDRs and
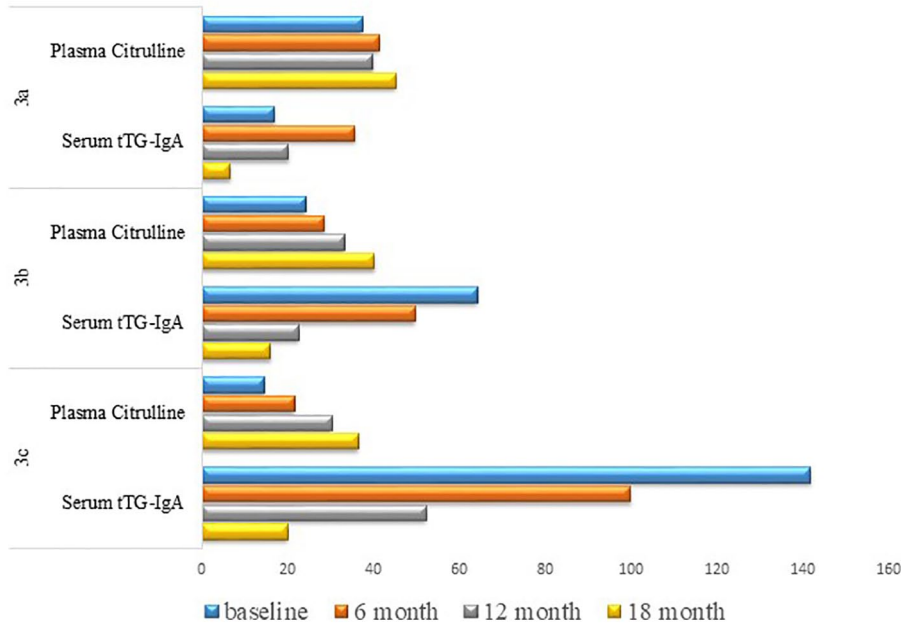
Figure 3. Depicts the decreasing serum tTG-IgA levels and increasing plasma citrulline levels from the diagnosis of CD to the 18 months followup on GFD. An inverse correlation was also observed in between citrulline and tTG-IgA levels at Marsh grade 3b and 3c.
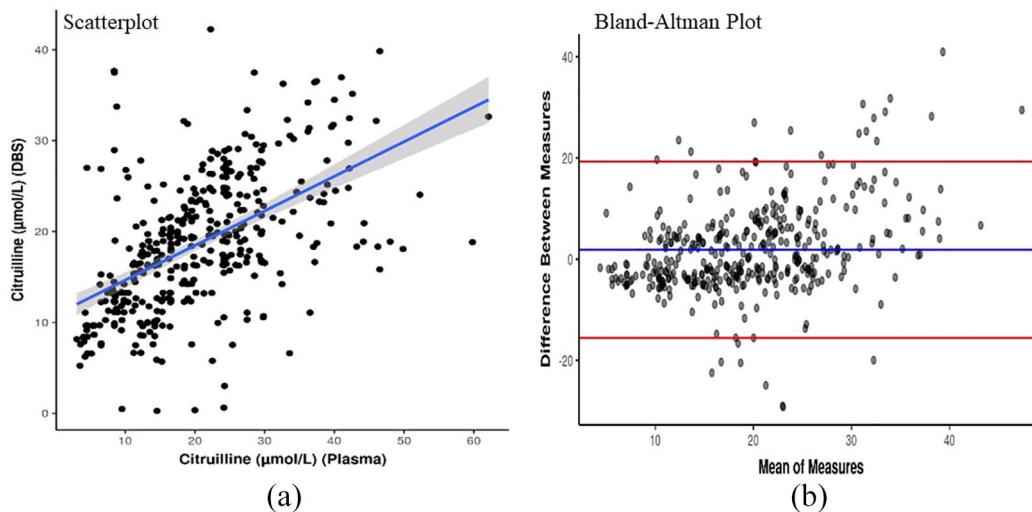

(a)   (b)

Figure 4. (a) Depicts the correlation between plasma citrulline and DBS citrulline levels. Individual points represent individual cases. Blue trend line represents the general trend of correlation between the 2 variables. Shaded gray area represents the 95% confidence interval of this trend line, and (b) depicts the comparison of the mean of 2 measures (*x*-axis) to the difference between the 2 measures (*y*-axis). The blue line represents the mean of the difference between the 2 measures, and the red lines represent the limits of agreement (mean±2SD of difference). The agreement was 94.8%.

its correlation with HLA genotype. In view of its efficacy to delineate the transition, we suggest its utility in predicting the progression of potential CD from asymptomatic to manifest CD over a 2-year period.

We also report the median plasma citrulline levels in children with CD to be 20.1 μMol/l (IQR, 13.35-29.15) and 37.33 μMol/l (IQR, 29.8-42.6) in controls with a statistical highly significant difference ($P < .001$). The large difference in the median values with non-overlapping inter quartile ranges (IQRs) suggest that these values can be used to differentiate between symptomatic CD and asymptomatic CD cases with a well defined disease range. Our findings are in sync with an

earlier study by Blasco et al[27] who also reported low plasma citrulline levels in children with CD (17.7 μMol/l) versus (28.9 μMol/l) in healthy controls. Sevinc et al[28] also described a significant difference ($P < .05$) in plasma citrulline between children (25 μMol/l) with CD and healthy controls (58 μMol/l). Basso et al[29] did not report any significant difference in plasma citrulline levels in CD patients on GFD (32.3 ± 8.7 μMol/l) and in healthy controls (33.8 ± 7.3 μMol/l) but suggested higher sensitivity of citrulline to pick mucosal atrophy in children <2 years. In a recent study from India, Singh et al[30] compared the citrulline between adult CD (16.1 μMol/l, IQR-7.7-27.7) and healthy adult controls (73.9 μMol/l,

51.5-123), ($P < .001$). Author reported a cut off value of ≤30 μMol/l as predictive with a sensitivity of 78.6% and specificity of 95.5% to detect villus abnormalities of Marsh 2 and above.

In patients not willing to undergo biopsy, ESPGHAN guidelines suggest the use of tTG-IgA levels more than 10 times of upper level of normal (ULN) range and positive anti endomysial antibody titers.[17] Correlation of tTG-IgA levels with small intestine histopathological changes confirmed that alone tTG-IgA marker is sufficient to predict the extent of the severity of Marsh grades.[31] Being an IgA assay with the lacunae of not being suggestive in case of concomitant IgA deficiency, we suggest citrulline to complement or replace tTG-IgA. It can also be used as a surrogate marker which can correlate with or predict tTG-IgA levels, severity of intestinal mucosal lesions and can also be utilized as a powerful tool for monitoring purposes. We noted an inverse correlation between plasma citrulline and serum tTG-IgA levels and observed an increasing trend of plasma citrulline levels with decreasing trends of serum tTG-IgA levels at different Marsh grades. Healing of damaged mucosa relates with the production of citrulline and is extensively dependent on gut enterocyte mass. When we correlated plasma citrulline levels with serum tTG-IgA level in patients with serum tTG-IgA levels less than 10 times of ULN and non-conclusive biopsy findings, we noted that in the absence of concrete biopsy diagnosis, plasma citrulline levels adequately reflected the image of damaged mucosal lining with the levels of 12.26 μMol/l. These levels were less then what we proposed (< 25.26 μMol/l) as the threshold for mucosal damage. Similar results were also noted in the patients with potential celiac disease when the biopsy findings were normal but median plasma citrulline levels were 23.25 μMol/l. The utility of citrulline as a marker and also in prediction of potential CD was best when evaluated in context of mucosal damage of grade 3b and above. Citrulline as assessed in our cohort, could effectively distinguish grades 3b and above but was not efficient in distinguishing 3a or less from CD patients and healthy asymptomatic FDRs, suggesting it to be a good predictor of damaged villous architecture in higher Marsh grade. Best diagnostic accuracy with AUC of plasma citrulline was observed with Marsh grade 3c mucosal lesions along with low negative predictive value also suggests the utility of citrulline in the diagnosis of CD with higher mucosal damage but may not be applicable in doubtful cases with Marsh grade 1 or 2 lesions. Moreover, a slight increase in the antibody level with low levels of citrulline in cases with increased intestinal permeability envisage that citrulline is not a specific marker or a signature for CD and elucidate the fact that citrulline estimation is not useful for the screening of doubtful cases with lower Marsh grades.

There is sparse data on the use of citrulline in identification of potential CD FDRs over a period of 24 months. We reported concomitant plasma citrulline and tTG-IgA values analyzed over 18 to 24 months period from baseline. An inverse correlation was observed with increasing plasma citrulline levels with declining tTG-IgA values (Table 3). This suggests that the regenerative capacity of the small bowel is restored with the adherence to GFD over a period of 6 months to a year. Crenn et al[25] reported that plasma citrulline levels were significantly increased in 6 GFD responsive patients. Levels at baseline were 17 ± 7 μMol/l and 31 ± 5 μMol/l after 1 year ($P < .01$). Miceli et al[32] also reported an increase of mean serum citrulline in CD patients after 2 years of the introduction of GFD (12.4 ± 8.3 μMol/l to 20.5 ± 10.9 μMol/l). Ioannou et al[33] reported that tTG-IgA antibody levels normalized after 1 year in the majority of patients and reported the declining pattern of anti tTG-IgA. Authors also reported an increasing trend of plasma citrulline levels on GFD from baseline (24.5 ± 4.9 μMol/l) to 1 month (27.6 ± 4.8 μMol/l) ($P < .043$). The levels were stabilized at 3 month (29.8 ± 5 μMol/l) and further increase was not seen at 6 month (30.4 ± 4.2 μMol/l) and 1 year (31.3 ± 5.9 μMol/l) on GFD. Our data on the contrary depicts a trend of increasing median plasma citrulline levels till 18 months on GFD.

Citrulline is a non HLA marker and its additive value over HLA genotype was further explored. Our data clearly depicts that plasma citrulline can accurately differentiate between serology negative and serology positive FDRs irrespective of their HLA genotypes to overcome the limitation of HLA testing due to 22% to 47.7% prevalence in healthy population. The haplotype being frequent in not affected population cannot be used in patient selection amongst high risk FDRs for monitoring purposes.[34-36] Plasma citrulline testing however, cannot replace HLA testing as it is a genetic susceptibility marker for the exclusion of low or no risk FDRs. Citrulline testing moreover can estimate the extent/severity of mucosal damage and kept its promise in differentiating healthy FDRs with high antibody levels than those with low levels. Citrulline testing is a better and more cost effective option compared to HLA testing as HLA testing is also not widely available in all centers. Assessing the utility of estimating citrulline collected on DBS is, easy transportation, storage, and low mail posting cost of DBS filter paper across the country. A statistically significant difference was noted between the presences of HLA genotypes with the levels of citrulline.

We observed that low plasma citrulline levels were associated with HLA DQ2.5 genotype ($P < .001$) with subtypes DQA1*0501 and DQB1*0201 respectively. This observation strengthens the risk assessment of above HLA genotypes. Patients with HLA DQ2.5 genotype have a high risk for the development of CD[37] and low levels of plasma citrulline in those patients elucidates the fact that these patients have a higher degree of mucosal damage. Different studies reported the prevalence ranges of HLA DQ2 and DQ8 genotype in CD patients between 73% to 95%.[38,39] This implies that 5% to 30% CD cases who do not possess HLA DQ2 and DQ8 genotypes can also develop CD. Dual testing of HLA genotype in

conjugation with plasma citrulline excludes no risk FDRs with normal mucosa. Additionally if we monitor plasma citrulline assay at follow ups in cases with absence of CD associated HLA genotypes, we can actually predict the tendency of disease development in these individuals. No significant difference was observed among FDRs with CD and controls in the distribution of citrulline levels in respect to HLA genotype. This could be attributed to the fact that a significant proportion (46.46%) of cases from suggestive biopsy corresponded with histological grade of Marsh 3a where citrulline levels were uninformative.

We observed a 94.8% agreement between the 2 measures, that is, 94.8% of the observations had a difference, which was within the limits of agreement ($\pm$17.43) between plasma and DBS estimation of citrulline. DBS measurement was in agreement in nearly 95% cases making it a reliable marker. DBS analysis involve recovery of analytes during elution and derivatization hence even a moderate agreement is suggested as good. The minimal blood sampling required for collection on DBS is good as it can be easily collected by a paramedical health worker. Citrulline estimation on DBS using Tandem mass spectrometry (TMS) is a minimally invasive and a promising test with the additional advantage of ease of transport, though the technological resource requires additional cost and expertise.

Though our study report one of the largest cohort of cases with CD and their FDRs but has some limitations. This study was conducted in a tertiary health care center, so its applicability in community settings is difficult. We did not rule out concomitant IgA deficiency which may have underestimated the prevalence accounting for approximately for 2% to 6% of total missed cases. Recruitment of the healthy, asymptomatic, serology negative FDRs as controls for the study could be considered as another potential limitation. Normative values may vary in children as well as in adults as the subgroup included all age groups hence, this would need consideration. Adherence to GFD was monitored clinically by growth parameter assessment on specialty days and failure of telephonic response and was not followed by a home visit so, this was responsible for attrition. We used only tTG-IgA analysis for serological compliance assessment. Usage of advanced assays like gluten immunogenic peptide detection in feces would have overcome this limitation.[40]

Conclusions

Our study provides evidence from a large sample size and emphasizes the utility of citrulline as a non-invasive, metabolomic biomarker for enteropathy in CD with a reliable sensitivity and specificity. Citrulline appears to be a promising assay in conjunction with HLA haplotypes and serological tests to diagnose, prognosticate, and evaluate adherence to GFD. This analyte has predictive value in high risk subclinical and potential FDRs of an index case with CD, irrespective of the dietary intake. There is a satisfactory agreement between DBS based analysis done by LCMSMS and plasma-based analysis as done by HPLC as DBS has ease of collection and transportation. Its validity for follow up in conjunction with HLA haplotype in first degree relatives is a significant outcome of our study but may need larger cohort for validation.

Author Contributions

Conceptualization, Seema Kapoor; Methodology, Avinash Lomash, Seema Kapoor.; Validation, Avinash Lomash; Formal analysis, Avinash Lomash, Dhwani Dholakia, Rishi Gupta.; Investigation, Avinash Lomash, Somesh Kumar, and Vinita Batra; Resources, Raghvendra Singh, Seema Kapoor, Vinita Batra, Praveen Kumar, Amarender S Puri; Writing: Original draft preparation, Avinash Lomash.; Writing: review and editing, Avinash Lomash, Raghvendra Singh, Seema Kapoor; Visualization, Avinash Lomash, Raghvendra Singh, Seema Kapoor; Supervision, Seema Kapoor, Amarender S Puri, Praveen Kumar, Anupa Prasad.

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

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