Serum Citrulline and Ornithine: Potential Markers of Coeliac Disease Activity

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ABSTRACT

Introduction: To date, there is not generally accepted and universal indicator of activity, and functional integrity of the small intestine in patients with coeliac disease. The aim of our study was to investigate whether serum concentrations of the non-essential amino acids citrulline and ornithine might have this function.

Methods: We examined serum citrulline and ornithine concentrations in a subgroup of patients with proven coeliac disease and healthy controls (blood donors).

Results: A total of 94 patients with coeliac disease (29 men, mean age 53 ± 18 years; 65 women, mean age 44 ± 14 years) and 35 healthy controls (blood donors) in whom coeliac disease was serologically excluded (10 men, mean age 51 ± 14 years; 25 women, mean age 46 ± 12 years) were included in the study. Significantly lower concentrations of serum ornithine were found in patients with coeliac disease (mean 65 ± 3 µmol/L; median 63 µmol/L, IQR 34 µmol/L, $p < 0.001$). No statistically nor clinically significant differences were found in the citrulline concentrations between the study and control group.

Conclusions: Serum ornithine (but not citrulline) may be useful for assessing the functional status of the small intestine in uncomplicated coeliac disease. Further studies involving more detailed analysis of dietary and metabolic changes in patients will be needed to reach definitive conclusions.

KEYWORDS

coeliac disease; citrulline; ornithine

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INTRODUCTION

Coeliac disease is a chronic, immune-mediated, and gluten-associated disease in genetically susceptible individuals (1, 2). The term "gluten-associated" more precisely describes its multisystemic nature. Other diseases may be associated with gluten intake too (3), which in fact, among the other things, makes the research and results interpretation difficult (4). Coeliac disease is a relatively common disorder with an average prevalence in Europe of 1% and in the Czech Republic up to 1 : 200 (2, 4, 5). On the contrary, mainly due to the frequent oligosymptomatic or asymptomatic course, coeliac disease is poorly considered in diagnostic algorithms and it is estimated that it is diagnosed early and correctly only in 10–15% of cases (6, 7).

The diagnostic approach in routine clinical practice is based on the determination of serum levels of antibodies against tissue transglutaminase (IgA class) and histological examination of biopsy samples of duodenal mucosa (1, 2, 4, 8). Small intestinal involvement plays a dominant role in the diagnosis and clinical manifestation of disease (1, 9). The characteristic histological picture of coeliac disease is an increased number of intraepithelial lymphocytes, crypt hyperplasia and especially atrophy of the small intestinal villi (10, 11). Villous atrophy is not specific to coeliac disease and may accompany several other enteropathies (1, 8). Although usually uncomplicated forms of coeliac disease do not primarily lead to significant intestinal dysfunction (12, 13), the disease can lead to malabsorption syndrome, including iron, trace element and vitamin deficiencies, and malnutrition. When intestinal function is reduced below the minimum necessary for absorption of macronutrients and/or water and electrolytes, so that intravenous supplementation is required to maintain health and/or growth, we speak of intestinal failure (12, 14–18). Patients with coeliac disease have an increased risk of developing serious complications – refractory coeliac disease, ulcerative jejunoileitis and/or small intestinal malignancies. In the presence of at least one of the above-mentioned conditions we speak about "complicated" coeliac disease and in their absence of "uncomplicated" coeliac disease (4, 19, 20).

A reliable indicator for assessing small bowel function is not yet available in patients with uncomplicated coeliac disease (4). One possible candidate marker is citrulline. It is a non-essential, non-protein (non-biogenic) amino acid whose biological potential was thought to be low for many years and has been identified in the human body only as an intermediate in the urea cycle and amino acid metabolism. Since 2000, some data have appeared in the literature on the role of circulating citrulline as a possible marker of functional integrity of the small intestine and villous atrophy in various enteropathies, including coeliac disease (21, 22). The role of citrulline in the body has been found to be complex, including its effect on the cardiovascular system (23), its antioxidant and immunomodulatory effects (24, 25), and especially its role in maintaining nitrogen homeostasis (26, 27). Interesting in this context is also the question of the possibility of using some of the precursors of citrulline, which are part of its synthesis in the enterocyte, for example ornithine or arginine and its correlation with citrulline levels. Clear data in this area are still lacking in the literature.

MATERIAL AND METHODS

The project was designed as a case-control study comparing a group of patients with previously diagnosed coeliac disease and healthy volunteers (blood donors). Inclusion criteria were a diagnosis of coeliac disease based on a combination of positive serology (always including anti-transglutaminase IgA) and histology. All patients underwent upper endoscopy with duodenal biopsy sampling at the time of initial diagnosis.

In control group of healthy donors, the coeliac disease was serologically excluded by means of negative results of multiple serum antibodies (anti-tissue transglutaminase IgA and IgG, anti-endomysial IgA and IgG, DGP (anti-deamidated gliadin peptide) IgA and IgG and anti-reticulin IgA and IgG).

A venous blood samples from patients and healthy controls (irrespective of fasting) were collected on a separation gel followed by centrifugation (2000 g gravity, 20 degrees Celsius) and freezing (–80 degrees Celsius) until final analysis. Then, an ultrahigh-performance liquid chromatography (Ultimate 3000 RS pump Dionex, Thermo Scientific, San Jose, CA, USA) coupled with high-resolution mass spectrometer with orbitrap analyser (Q-Exactive Focus, Thermo Scientific, San Jose, CA, USA) was used in quantitative analyses of citrulline and ornithine in serum. Sample preparation was based on previously published method using pentafluorobenzoyl chloride as derivatization reagent (28). Isotopically labelled citruline-d_4 and ornithine-d_6 were used as internal standards. Chromatographic separation with mass spectrometry analysis was developed and optimized, followed by full method validation according to the Guideline on Bioanalytical Method Validation (29). All evaluated parameters met the required criteria proving the method to be precise and accurate. Certified lyophilised calibration and control materials (RECIPE Chemicals + Instruments GmbH, Munich, Germany) were used during study.

The obtained data were processed using methods of descriptive statistics. Data by normal distribution of values were further analysed by parametric two-sample t-test and the data with non-normal distribution were tested by non-parametric Mann-Whitney and Kolmogorov-Smirnov test. For qualitative parameters the Fisher's exact test was used. For correlation analysis the Spearman's rank correlation coefficient was used. For the above-mentioned statistical analysis was used NCSS 2021 Statistical Software (NCSS, LLC, Kaysville, Utah, USA, 2021: ncss.com/software/ncss). The study was approved by the Joint University Ethics Committee (Reference number: 202 107 PO6). All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its latter amendments (30). All patients signed written consent. For all data obtained, all personal identification information were deleted in compliance with the laws for the protection of confidentiality of the Czech Republic.

RESULTS

Two subgroups entered the study: the study group of 94 patients with coeliac disease and the control group of 35 healthy blood donors. The age and sex structure of the study group was 29 men (31%), mean age 53 ± 18 years (median: 47 years, interquartile range: 31 years) and 65 women (69%), mean age 44 ± 14 years (median: 41 years, interquartile range: 20 years) – details in Table 1. The age and gender structure of the controle group consisted of 10 men (29%), mean age 51 ± 14 years (median 47 years, interquartile range 19 years) and 25 women (71%), mean age 46 ± 12 years (median 45 years, interquartile range 18 years) – details in Table 1.

The mean disease duration (in the group of coeliac disease patients) was 160 ± 144 months, median: 118 months (interquartile range: 181 months). The most common histological finding in coeliac disease patients was type 3a according to the Marsh-Oberhuber classification. None of the patients underwent small bowel resection and/or splenectomy. Some autoimmune-associated diseases occurred in the group of patients: rheumatoid arthritis (1 case), autoimmune thyreopathy (19 cases), atopic dermatitis (4 cases), vitiligo (3 cases), autoimmune hepatitis (1 case), IgM nefropathy (1 case), type 1 diabetes mellitus (5 cases), asthma bronchiale (12 cases), polyserositis (1 case), antiphospholipid syndrome (1 case), systemic sclerosis (2 cases), autoimmune gastritis (1 case), spondylarthritis (1 case), psoriasis (2 cases), eosinophilic oesophagitis (1 case), hereditary angioedema (1 case). There was no patient with associated Sjögren syndrome. IgA deficiency was found in four patients and in no healthy control. HLA genotyping was not performed in either patients or healthy controls. The patient did not include a person with current acute (or acute on chronic) kidney injury that could affect serum citrulline and/or ornithine concentrations.

In a statistical survey of patients with coeliac disease and group of healthy controls (blood donors), their quantitative and qualitative features were monitored and compared. In the monitoring of quantitative traits, emphasis was placed on serum concentrations of citrulline and ornithine as possible indicators of small intestinal function and total enterocyte mass.

Serum ornithine concentrations showed a statistically significant differences between the group of patients with coeliac disease and the group of healthy controls ($p < 0.001$). The statistically significant differences were identified in serum transglutaminase IgA concentration ($p = 0.024$) too (other monitored clinical and laboratory

Tab. 1 Basic quantitative features in patients with coeliac disease and healthy controls (blood donors). SD – standard deviation, NS – not significant, S – significant, µmol – micromol, U – unit, L – litre, IQR – interquartile range, n = number of subjects, BMI = body mass index, BSA = body surface area, MCV = mean corpuscular volume.

Parameter	Controls (n = number of subjects; mean ± SD; median; IQR)	Coeliac disease (n = number of subjects; mean ± SD; median; IQR)	Statistical significance
Age (years)	n = 35 47 ± 13 47; 18	n = 94 46 ± 16 43; 21	NS (p = 0.372)
Weight (kilograms)	n = 35 82 ± 22 75; 23	n = 94 70 ± 16 68; 21	NS (p = 0.062)
BMI (kg/m$^{2)}$	n = 35 27 ± 4 27; 6	n = 94 25 ± 5 24; 6	S (p = 0.006)
BSA (m$^{2)}$	n = 35 1,8 ± 0,2 1,8; 0,3	n = 94 1,7 ± 0,2 1,7; 0,3	S (p = 0.011)
Haemoglobin (g/L)	n = 35 142 ± 10 141; 13	n = 94 137 ± 13 138; 15	NS (p = 0.072)
Leukocytes (10^{9}/L)	n = 35 7 ± 2 6; 2	n = 94 7 ± 2 7; 3	NS (p = 0.814)
MCV (fL)	n = 35 90 ± 4 91; 5	n = 94 87 ± 5 87; 5	S (p = 0.002)
Transglutaminase IgA (U/mL)	n = 35 2 ± 2 2; 1	n = 88 12 ± 42 3; 4	S (p = 0.024)
Citrulline (µmol/L)	n = 35 30 ± 10 29; 10	n = 94 27 ± 10 27; 15	NS (p = 0.136)
Ornithine (µmol/L)	n = 35 97 ± 26 97; 42	n = 94 65 ± 3 63; 34	S (p < 0.001)

markers in Table 1). Due to the mostly rejected normality, the median and interquartile ranges (75th percentile minus 25th percentile) were used to express the degree of variability in addition to the arithmetic mean and standard deviation values. Correlation analysis using the nonparametric Spearman's rank correlation coefficient proved statistically significant stronger correlation between plasma citrulline and ornithine concentrations in the group of patients with coeliac disease (Spearman's coefficient = 0.723) versus the group of healthy controls where the correlation was weak (0.723 vs. 0.313; Table 2, Figure 1) (31). We did not find a strong correlation between disease duration and serum citrulline and / or ornithine concentrations.

For other quantitative features no strong correlation rate was found in the group of patients with coeliac disease and healthy controls (Table 3).

The main qualitative characteristic monitored was smoking. There was a total of 13 smokers (14%) in the group of patients (5 men (39%), 8 women (61%)), and 5 smokers (14%) in the control group (2 men (40%), 3 women (60%)). We did not find any significant differences in the serum concentrations of citrulline and ornithine in smokers and non-smokers in the two groups studied (Table 4 and 5).

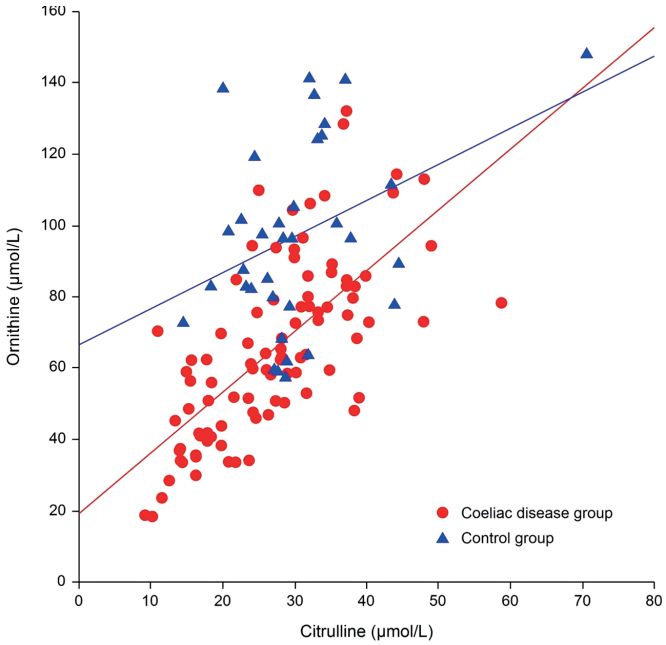

Fig. 1 Correlation of serum citrulline and ornithine concentrations in the group of patients with coeliac disease (Spearman's coefficient = 0.723) and healthy controls (Spearman's coefficient = 0.313).

Tab. 2 Correlation between other quantitative traits and serum concentrations of citrulline and ornithine in the group of coeliac patients (expressed by Spearman's coefficient). M – males, F – females, SD – standard deviation, µmol – micromol, U – unit, L – litre, IQR – interquartile range, n = number of subjects.

Parameter	**Citrulline – total** (M / F)	**Correlation strength**	**Ornithine – total** (M / F)	**Correlation strength**
Age (years)	0.215 (0.081 / 0.261)	weak (very weak / weak)	0.303 (-0.002 / 0.431)	weak (very weak /medium)
Weight (kilograms)	-0.066 (-0.079 / -0.105)	very weak (very weak / very weak)	0.093 (0.007 / 0.082)	very weak (very weak/ very weak)
BMI (kg/m^2)	0.027 (0.126 / -0.001)	very weak (very weak / very weak)	0.138 (0.149 / 0.124)	very weak (very weak/ very weak)
BSA (m^2)	-0.132 (-0.185 / -0.169)	very weak (very weak / very weak)	0.072 (-0.062 / 0.055)	very weak (very weak / very weak)
Haemoglobin (g/L)	0.111	very weak	0.042	very weak
Leukocytes (10^9/L)	-0.0445	very weak	-0.074	very weak
MCV (fL)	-0.064	very weak	0.028	very weak
Transglutaminase IgA (U/mL)	-0.206	weak	-0.065	very weak
Disease duration (months)	-0.0305	very weak	0.0047	very weak

Tab. 3 Correlation between other quantitative traits and serum concentrations of citrulline and ornithine in the group of healthy controls (expressed by Spearman's coefficient). M – males, F – females, SD – standard deviation, µmol – micromol, U – unit, L – litre, IQR – interquartile range, n = number of subjects.

Parameter	**Citrulline –total** (M / F)	**Correlation strenght**	**Ornithine – total** (M / F)	**Correlation strenght**
Age (years)	0.375 (0.610 / 0.204)	weak (strong / weak)	0.132 (-0.553 / 0.345)	very weak (medium / weak)
Weight (kilograms)	0.101 (-0.330 / 0.073)	very weak (weak / very weak)	0.119 (-0.128 / 0.144)	very weak (very weak / very weak)
BMI (kg/m^2)	0.027 (-0.430 / 0.002)	very weak (medium / very weak)	0.232 (0.139 / 0.233)	weak (very weak / weak)
BSA (m^2)	0.128 (-0.321 / 0.070)	very weak (weak / very weak)	0.221 (-0.079 / 0.284)	weak (very weak / weak)
Haemoglobin (g/L)	0.105	very weak	-0.125	very weak
Leukocytes (10^9/L)	-0.510	medium	-0.245	weak
MCV (fL)	0.092	very weak	-0.086	very weak
Transglutaminase IgA (U/mL)	-0.175	very weak	-0.027	very weak

Tab. 4 Serum concentrations of citrulline and ornithine in the group of patients with coeliac disease in relation to smoking. SD – standard daviation, NS – not significant, S – significant, µmol – micromol, L – litre, IQR – interquartile range, n = number of subjects.

Parameter	Smoking – YES (n = number of subjects; mean ± SD; median; IQR)	Smoking – NO (n = number of subjects; mean ± SD; median; IQR)	Statistical significance
Citrulline (µmol/L)	n = 13 26 ± 10 22; 15	n = 81 27 ± 10 27; 15	NS (p = 0.579)
Ornithine (µmol/L)	n = 13 69 ± 29 62; 41	n = 81 65 ± 24 63; 33	NS (p = 0.550)

Tab. 5 Serum concentrations of citrulline and ornithine in a group of healthy controls in relation to smoking. SD – standard daviation, NS – not significant, S – significant, µmol – micromole, L – litre, IQR – interquartile range, n = number of subjects.

Parameter	Smoking – YES (n = number of subjects; mean ± SD; median; IQR)	Smoking – NO (n = number of subjects; mean ± SD; median; IQR)	Statistical significance
Citrulline (µmol/L)	n = 5 28 ± 4 28; 7	n = 30 31 ± 10 29; 11	NS (p = 0.588)
Ornithine (µmol/L)	n = 5 97 ± 29 98; 50	n = 30 97 ± 26 97; 43	NS (p = 0.948)

DISCUSSION

The aim of this study was to investigate the significance of serum amino acids citrulline and ornithine as potential non-invasive markers of small intestinal damage in patients with coeliac disease. The main finding of our study is statistically significantly lower serum ornithine concentrations in patients with uncomplicated coeliac disease compared to healthy controls. On the other hand, no statistically significant difference in serum citrulline concentrations was found.

The diagnosis of coeliac disease is currently well defined, based on a combination of positive serological markers (positive antibodies to tissue transglutaminase IgA, TTG IgA) and a typical histological picture in the small intestinal mucosa (1, 2, 4, 8, 10, 11). On the other hand, there is still no reliable indicator of celiac disease activity to monitor patients (4). In real clinical practice, the "gold" standard of disease monitoring remains the determination of specific autoantibodies and invasive histological examination of biopsy samples from duodenal mucosa (1, 2, 4, 8) – however, this approach has several problematic moments and, in some cases, fails. The serological activity of coeliac disease-specific autoantibodies may be difficult to interpret in immunocompromised patients (e.g. immunodeficiency conditions including IgA deficiency, immunomodulatory treatment for another diagnosis). Other areas reducing the usefulness of serological markers in monitoring coeliac disease activity are immune dysregulation, as evidenced by the frequently associated functional hyposplenism and selective memory B-lymphocyte deficiency (32) and the so-called seronegative coeliac disease (4) with the inability of TTG IgA to leave the mucosa into the bloodstream (33). Moreover, the dynamics of the serological response can be misleading. Although a reduction in serum TTG IgA concentrations indicates the effect of a gluten-free diet and is a marker of reduced disease activity (34), an antibody response (and thus serologic detection of TTG IgA) may not be present when less gluten is re-consumed.

In these cases, TTG IgA sensitivity decreases and does not reflect the severity of histological changes in the small intestinal mucosa (8). Even changes in histological findings in the small intestine may not be a reliable indicator of the course of the disease. Histological changes mimicking coeliac disease occur e.g. with commonly available and used drugs (NSAIDs, PPIs). Moreover, histological monitoring is a relatively invasive approach. In view of this, intensive efforts are being made to find new non-invasive markers of the functional status of the small intestine.

The first findings of the biological nature and function of citrulline and its relationship to the small intestine were provided by Windmüller et al. (35). Since 2000, Crenn et al. presents citrulline as a possible laboratory indicator of intestinal failure and diseases associated with villous atrophy (21, 22). There is a strong correlation between serum citrulline levels and the severity of small bowel failure (which is well and precisely defined), but the diagnosis of intestinal failure remains relatively rare (12, 17) and uncomplicated coeliac disease is not a common cause of intestinal failure (13, 15). Although citrulline has been the subject of other published studies (22–40) describing its relationship to intestinal diseases (plasma concentrations reliably reflect the total functional mass of enterocytes) (22, 36–41), its determination is not well defined in routine clinical practice. An important precursor of citrulline in enterocytes is ornithine, whose role in relation to intestinal diseases is unclear (42, 43). Citrulline is found in the body in two forms (free citrulline and citrullinated proteins) (44), and the main site of its synthesis from dietary arginine, glutamine and proline (via ornithine as a direct precursor) is the small intestine (mainly the abo-

ral ileum) (35, 42, 44). In this context, it should be noted that enterocytes are equipped with transport systems that also allow efficient uptake of luminal citrulline (45, 46). Citrulline is released from enterocytes into the portal circulation (not taken up by the liver) and continues to the cells of the proximal renal tubule, where undergo its final conversion to arginine (35, 42, 47, 48). Arginine is then available through the systemic circulation to the whole body for proteosynthesis. This metabolic pathway (arginine / glutamine – ornithine – citrulline) plays an important role in maintaining the body's nitrogen homeostasis (42). Citrullin is involved in the post-translational modification of some proteins (citrullination) (48) and several diseases (e.g. rheumatoid arthritis, psoriasis and multiple sclerosis) are associated with the presence of citrullinated proteins or antibodies against them (49–54). Ornithine is involved in complex metabolic processes (regulation of growth hormone production, lipolysis and immunomodulation) and the urease cycle (37, 55).

Literature data suggest the utility of plasma citrulline levels to monitor disease severity in patients with extensive and/or destructive involvement of the small intestinal mucosa (including complicated forms of coeliac disease such as refractory coeliac disease, ulcerative jejunoileitis and T-cell lymphoma-associated enteropathy), which can lead to small bowel failure and/or malabsorption syndrome (12, 13), rather than for patients with uncomplicated forms of coeliac disease (22, 56). All patients included in our study had previously diagnosed coeliac disease and were treated with a gluten-free diet with significant clinical effect (no patient had clinically active disease). None of the patients had laboratory evidence of intestinal failure or malabsorption syndrome. Although we found some statistically significant differences in body mass index (BMI, $p = 0.006$), body surface area (BSA, $p = 0.011$) and mean corpuscular volume (MCV, $p = 0.002$) between patients with coeliac disease and healthy controls, these findings could not be considered clinically significant (given the relatively low values) – Table 1. Considering this, and completely in accordance with the literature data, no statistically significant difference in serum citrulline levels was found between coeliac patients and healthy controls in our study.

Although the statistically significantly lower plasma concentrations of ornithine found in our study in patients with uncomplicated coeliac disease ($p < 0.001$) compared to healthy controls might suggest its greater sensitivity compared to citrulline as a marker of small intestinal mucosal damage, other explanations for this finding have been offered. One explanation may be changes in serum levels of various amino acids caused by differences between individuals on gluten-free and regular diets (blood donors in our cohort). Citrulline is a specific product of enterocytes and is not present in the diet (with the exception of melons) (57) and is not released by other cells (both study groups, i.e., coeliac patients and a control group of healthy blood donors, were not burdened by a diet containing melons). In contrast, ornithine is common in the diet and its daily intake is a few grams. However, an important fact is its association with another amino acid, arginine, which is a substrate for the formation of ornithine. It is the reduced dietary intake of arginine in patients on a gluten-free diet that may theoretically contribute to lower serum ornithine levels (58). From this perspective, a possible effect of fasting on serum ornithine levels cannot be excluded. However, the levels of both amino acids (citrulline and ornithine) were examined in our study in non-fasting patients and/or healthy controls. In addition, gluten itself stimulates the activity of arginase (the enzyme required to convert arginine to ornithine), and therefore a gluten-free diet may also lead by this mechanism to lower production and reduced serum ornithine concentrations (59).

It is a question (or rather speculation) whether the above statements can explain the surprisingly statistically significant correlation between plasma concentrations of citrulline and ornithine in the group of patients with coeliac disease compared to the group of healthy controls, where the correlation was weak (Table 2, Figure 1). In view of this, it will be necessary to verify our data by a more detailed analysis of the dietary amino acid content and metabolic changes in their production.

Our study has some important limitations. The main one is the lack of longitudinal monitoring of the observed changes in serum amino acid levels over time and the lack of correlation with invasive tests (histological changes in the small intestinal mucosa) and more detailed nutritional parameters analysis (including serum iron levels, ferritin, etc.). A minor limitation is the absence of HLA genotyping in the group of healthy controls to definitively exclude seronegative coeliac disease, although its probability in this group is low.

CONCLUSIONS

There is still no universal and generally accepted indicator of coeliac disease activity, especially in patients with uncomplicated coeliac disease. The non-essential amino acids citrulline and ornithine and their determination in serum seem to be suitable candidates. Serum ornithine might be more sensitive for assessing disease activity in patients with coeliac disease without intestinal failure and/or malassimilation syndrome. Further studies will be needed to confirm our results, especially focusing on the effects of diet and metabolic changes on the serum levels of specific amino acids.

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