This is the accepted manuscript of the article first published: 14 April 2020, in *Journal of Clinical Gastroenterology*. The final published version at: https://doi.org/10.1097/mcg.0000000000001349

Non-biopsy approach to celiac disease is accurate when using exact duodenal histomorphometry: a prospective study in two countries

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Abstract

Goals: To test the accuracy of serology-based criteria for diagnosing celiac disease utilizing quantitative histomorphometry.

Background: The revised European pediatric guidelines allow non-invasive celiac disease diagnosis for a subgroup of children. However, in some of the studies on this issue the positive predictive value (PPV) of serology has remained suboptimal, possibly due to challenges of histopathology as reference standard.

Study: Prospectively enrolled children with transglutaminase 2 antibodies (TGA) above the upper limit of normal (ULN) underwent blood sampling and duodenal biopsy in Finland and Romania. Those with TGA ≥10x ULN, positive endomysium antibodies (EmA), and disease-associated genetics were considered to fulfil triple criteria for celiac disease. Initial histopathological analysis was conducted using grouped classification, whereupon centralized morphometry was performed.

Results: Altogether 88 (54%) children were triple positive. In local evaluation, 99% of triple-positive children and 73% of children with TGA <10x ULN had celiac disease. These figures increased to 100% and 85% after more precise morphometric analysis. Triple-positive children had more anemia and higher median EmA and liver enzyme values than those with TGA <10x ULN; the groups were comparable in other clinical features and laboratory parameters.

Conclusions: When applied as recommended, the non-biopsy strategy had an already yielded excellent PPV regardless of the site of diagnosis or clinical presentation in local analysis. PPV further increased to 100% with standardized duodenal morphometry.

ClincialTrials.gov, NCT02072590

Keyword: diagnosis; serology; histomorphometry; biopsy

Introduction

Celiac disease is a common although underdiagnosed immune-mediated disease affecting up to 1-3% of pediatric population.[1,2] The gluten-driven condition is characterized by the presence of disease-specific serum autoantibodies to transglutaminase 2 (TGA) and endomysium (EmA).[3] For decades, demonstration of villous atrophy with crypt hyperplasia in duodenal biopsies has been the foundation of the diagnosis.[4,5] However, this histological finding is not pathognomic for celiac disease, and technical challenges with biopsy sampling and interpretation of the histology may lead to false positive or negative results.[6,7] Furthermore, gastrointestinal endoscopy is invasive and in children usually requires general anesthesia.

Owing to these challenges and improved serological tests, the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) in 2012 issued guidelines allowing a non-biopsy diagnosis in symptomatic children having TGA ≥10 times the upper limit of normal (ULN), positive EmA, and correct genetics.[8] In the most recent revision of the guidelines, genetic testing is not mandatory and asymptomatic children can also be diagnosed based solely on serology.[9] Two large prospective studies have supported this approach,[10,11] but in some of these the positive predictive value (PPV) has remained suboptimal,[12-16] and the criteria have not been officially accepted in the USA. One reason for the inconsistent results may be the use of error-prone histopathology as the reference standard. Beside the aforesaid technical challenges, the widely used grouped classification can be inaccurate in borderline cases.[13,17,18] Pitfalls in histologic examination are a frequent reason for false-negative biopsy in seropositive individuals.[6,10,17,18] In order to improve the diagnostics, we have developed validated morphometric procedures for precise duodenal histopathology.[7]

We here aimed to establish the accuracy of the non-biopsy strategy for celiac disease when optimized histology is used as a reference standard. This was accomplished by applying our standardized

morphometric procedures in children participating in a prospective study in two countries with markedly different socioeconomic environments and healthcare systems.

Material and Methods

Patients and study design

The study was conducted in tertiary and secondary centers in Finland (Department of Pediatrics, Tampere University Hospital, and Department of Pediatrics, Hämeenlinna Central Hospital) and in a tertiary center in Romania (National Institute for Mother and Child Health, Bucharest). The patient cohort comprised all children (age ≤16 years) with suspected celiac disease and TGA above the normal reference range who were willing to participate. Exclusion criteria were refusal to participate, negative celiac disease serology and initiation of a gluten-free diet before the diagnostic investigations.

All participants underwent a clinical evaluation and blood sampling and an upper gastrointestinal endoscopy with small-bowel mucosal biopsies. The biopsies were subsequently referred to local histopathological analysis and to centralized morphometric analysis. The specificity of the serology-based criteria for celiac disease was calculated for both approaches. Triple criteria for celiac disease were defined as TGA ≥10 times ULN, positive EmA, and presence of celiac disease-associated HLA. In addition, the children were divided into those with low (<10x ULN) and high (≥10x ULN) TGA values and the study variables were compared between these subgroups. In order to assess the possible effects of the site of investigations, all comparisons were repeated among children diagnosed in Romania and in Finland.

The study protocol and patient recruitment were approved by the Ethics Committee of Pirkanmaa Hospital Region, Tampere, Finland and the Ethics Committee of the University of Medicine and

Pharmacy “Carol Davila” and the National Institute for Mother and Child Health “Alessandrescu-Rusescu”, Bucharest, Romania. All participating children and/or their guardians gave written informed consent. All authors had access to the study data and have reviewed and approved the final manuscript.

Clinical information and laboratory parameters

Demographic and anthropometric data, clinical presentation including main reason for suspicion of celiac disease, and possible presence of chronic comorbidities and long-term medication were collected. The main presenting symptoms were further classified into gastrointestinal (e.g. diarrhea, vomiting, abdominal pain, constipation) and extraintestinal (e.g. poor growth, arthralgia, rash, and neurological symptoms). The reason for screening was registered for screen-detected children.

Standardized body mass index (BMI-SDS) was calculated as height/weight^2 adjusted for age and gender. Laboratory parameters measured with routine methodology included blood hemoglobin (Hb, lower reference value (Rf) from 95-117 g/l according to age and sex), erythrocyte mean corpuscular volume (MCV, Rf 73-95 fl), ferritin (Rf >10µg/l), alanine aminotransferase (ALT, Rf ≤30 U/L), and albumin (Rf 36-48 g/l).

Celiac disease serology and genetics

Serum IgA class TGA were determined using concentration-dependent enzyme-linked immunosorbent assay based on calibration curve (Celikey, Phadia GmbH, Freiburg, Germany). The ULN was set at 7 U/l as recommended by the manufacturer. Serum EmA were assessed by indirect immunofluorescence method using human umbilical cord as substrate.[19] Cut-off for EmA positivity was serum dilution 1:5 and positive samples were further diluted up to 1:4000. Quantitative

determination of total serum IgA was done for all children in order to detect possible IgA deficiency (<0.2 g/l), in which case the corresponding IgG class TGA and EmA were measured.

The presence of DQB1 and DQA1 alleles encoding celiac disease-associated human leukocytes antigen (HLA) DQ2 and DQ8 was assessed in whole blood samples using the SSPTM DQB1 low-121 resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) and/or tagging SNP approach.

Histological assessment

Duodenal sampling was carried out upon esophagogastroduodenoscopy. A minimum of two representative biopsy specimens was taken from the duodenal bulb and four specimens from the distal duodenum (D2/D3) and preserved in formalin. One sample was taken for each pass of the biopsy forceps. Histological evaluation was conducted in each clinical center after systematic orientation of the mucosal biopsies. Level section and change of cutting angle were applied during the preparation of the specimens. Analyses of possible mucosal inflammation and villous damage were done under light microscope from hematoxylin-eosin stained biopsy cuttings. The lesion was graded using Marsh-Oberhuber classification by three pathologists with expertise in pediatric alimentary tract.[20]

Besides the local histopathological analysis, the biopsies underwent a centralized morphometric evaluation of quantitative measurements of villous height-crypt depth ratio (VH/CrD). The measurements were done by two specifically trained readers, who in a previous validation study achieve an intraclass correlation coefficient of 0.978.[7] In VH/CrD analysis, both the small-bowel mucosal villi and crypts are essential for quantitative measurement. In cases where crypt depths could not be precisely evaluated over their entire length, the biopsies were further recut until optimally oriented sections amenable to measurement were obtained. The final VH/CrD was calculated as mean of at least three well-oriented villous-crypt units and a ratio of >2.0 was considered normal.[7,21] All

centralized analyses were carried out blinded without knowledge of previous medical history or celiac disease serology.

Some biopsy specimens were snap-frozen and used for further assessment of the small-bowel mucosal TG targeted IgA deposits, particularly in cases with inconsistent results between local and centralized histology. The presence of celiac disease-specific deposits was evaluated with immunohistochemistry from unfixed frozen sections by double-staining of the mucosal IgA and TG.[22,23]

Statistics

Statistical analyses were conducted using Epi Info for Windows software, version 7.2 (provided by Center for Surveillance, Epidemiology, and Laboratory Services, Georgia, USA). Categorized values are reported as numbers and percentages. Numerical parameters were either ordinal or markedly skewed and for the sake of simplicity they are thus all reported as medians with quartiles. Statistical significance of the differences between groups was tested using either Chi Square or Fisher's Exact test (categorized values) or Mann-Whitney U (numerical values) test as appropriate. The limit for statistical significance was set at P value <0.05.

Results

Basic characteristics of the study cohort

Altogether 182 children fulfilled the other inclusion criteria, but fifteen families refused to participate and three families withdrew. Thus, the final cohort comprised 164 subjects (Figure 1). Their median age was 7.0 (quartiles 4.5-11.7) years and 71% were girls. One child was IgA deficient and was found to have positive IgG class TGA. None of the participants had immunosuppressive or other medication or reduced gluten use possibly affecting the diagnostic findings.

Triple positivity and local diagnostics

Altogether 88 (54%) out of the 163 seropositive children with normal IgA had TGA values ≥10x ULN (Figure 1). All 88 had positive EmA and celiac disease-associated HLA and thus fulfilled the triple criteria. In the local histopathological evaluation, all but one (99%) of children with triple positivity and 55 (74%) out of the remaining 75 children with positive TGA <10x ULN were found to have celiac disease. EmA was positive in all but two subjects with TGA <10x ULN excluding one child for whom EmA was not available (Figure 1).

Centralized histology

In the centralized morphometric examination with VH/CrD, a histological outcome different from the local report was found in one child consistent with the triple criteria and in nine children with TGA <10x ULN; in each case the result changed from non-diagnostic to celiac disease (Table 1). All 10 subjects had TG-targeted mucosal IgA deposits. Consequently, 100% of children with triple positivity and 85% of those with positive TGA <10x ULN eventually fulfilled histopathologic criteria for celiac disease (Figure 1). The remaining 11 children with no celiac disease even after morphometric analysis had TGA level ≤ 5x ULN. Of these, EmA was positive in all nine and TG2-targeted IgA deposits in eight out of the ten children in whom these were measured. The two cases with negative deposits had TGA values of 7 U/l and 9 U/l respectively. Most of the children having only mild or no histological lesions remained under careful follow-up, but some started a gluten-free diet upon a joint decision with the family.

Comparison between children with low and high antibody titers

Children with TGA >10x ULN had less celiac disease in the family and borderline significantly more anemia than those with TGA <10x ULN, whereas the groups did not differ in demographic data, main reason for suspicion of celiac disease or presence of chronic comorbidities (Table 2). The triple-

positive group also had higher median EmA and ALT and lower median ferritin and MCV than those having TGA <10x ULN, while there were no significant differences in Hb, albumin, and BMI (Table 3).

Asymptomatic children

Six children from Romania and eight from Finland were reported to be asymptomatic. Six of these 14 had TGA >10xULN. One asymptomatic child from Romania and one from Finland were deemed non-celiac in the local pathology report but were found to have diagnostic lesions in morphometric analysis (Table 1). The other 12 patients were already deemed to have celiac disease in local evaluation.

Comparison between Romanian and Finnish children

In a separate analysis between countries, 23 out of 33 (70%) of Romanian children and 65 out of 130 (50%) of Finnish children were triple positive (Table 4). The Romanian children had fewer other chronic conditions and lower BMI and higher MCV, ferritin, and ALT values, whereas there were no differences between countries in other patient characteristics (Table 4).

Discussion

We found the ESPGHAN non-biopsy approach to have an excellent PPV for celiac disease in local centers when using serology and histology as recommended. The figure improved up to 100% when applying our standard operating procedures for quantitative morphometry. The result was affected neither by the variability in the initial patient characteristics nor by the site of the celiac disease diagnosis, further supporting the reliability of the criteria in all settings when compared to optimized histological reference.

The excellent PPV of high TGA levels for celiac disease already with the conventional grouped histology are in line with those reported in the recent multicenter studies by Werkstetter et al. and Wolf et al.[10,11] Retrospective studies have also been conducted and in most of these PPV has varied between 98 and 100%.[12,13,14,24] Although seemingly good, the lower end of these percentages could still be considered insufficient as with such a common disease their use would lead to a significant number of misclassified patients. Moreover, even markedly inferior results have been obtained; for instance, Trovato et al.[15] reported a PPV of 91% and Elitsur et al.[16] of only 88% for TGA ≥10x ULN. These results have raised concerns about the generalizability of the non-biopsy criteria to all populations and healthcare settings and could be one reason for the ongoing debate on their validity.

There could, however, also be methodological reasons for these suboptimal results. In many studies the non-biopsy criteria were not strictly adhered to or at least this was not reported; i.e. the use of TGA test with a known calibration curve, systematic measurement of EmA and HLA and careful histological analysis.[8] Even if correctly applied, several issues complicate testing the criteria. A major problem is the lack of standardization of TGA tests, preventing the setting of a fixed cut-off for omitting the biopsy. This has led to the introduction of a simple but "safe" cut-off of 10x ULN and the use of HLA and EmA to control the assay variation.[8,25,26] One explanation for low PPV in some studies could thus be inconsistent use of TGA cutoffs and omission of confirmatory testing.[25] When using validated serology as recommended, we found 100% agreement with the >10x ULN, positive EmA, and HLA. This has also been observed in other recent studies, indicating that high values of TGA would suffice for the diagnosis as such with a proven accurate test and, accordingly, the requirement of HLA was already removed from the updated ESPGHAN criteria.[9] In fact, we also found 100% PPV for celiac disease with TGA values >5x ULN, but the problem with non-standardization should likely be resolved before the use of serology-based diagnosis can be extended.[10,11]

There are also challenges in the use of histology as the diagnostic gold standard, including the presence of the lesions at only the very proximal part of the duodenum, patchiness of the lesion between or even within the biopsy, and poor quality and/or wrong orientation of the samples.[7,27,28,29,30] It again remains unclear whether these critical issues have been considered in all studies. Moreover, even if executed appropriately, the usually applied grouped classification have shown relatively poor interobserver agreement.[7,18,29] Accordingly, in the study by Werkstetter et al.[10], numerous original diagnoses changed after centralized histopathology and many remained inconclusive even in the second evaluation due to poor biopsy quality. These problems are likely accentuated in centers having less experience with celiac disease. We thus believe that initial testing of the serology-based criteria should be carried out in optimized research settings and thereafter, if proven accurate, they could serve as an easier and even more accurate diagnostic approach in daily practice.

Standardized duodenal morphometry improved the diagnostics particularly in children with low non-diagnostic TGA values and subsequently less advanced mucosal damage.[31] This proves that precise histopathology will remain as important as ever among those who still need the biopsy. In fact, in the absence of good orientation neither morphometry nor grouped classification (i.e., Marsh classes) can be accurate. It can be argued that morphometry is impractical in routine practice, but our results show that standardized procedures for orientation and measurement improve the diagnostics of inconclusive cases.[9] This also applies to special diagnostic methods, such as intestinal IgA deposits, which were assessed here mainly to confirm the diagnosis. With TGA values <5x ULN non-diagnostic cases began to emerge even after centralized evaluation. Interestingly, however, all these children were positive for EmA and HLA and almost all had IgA deposits. This concurs with our earlier studies and demonstrates the gradual development of the duodenal lesion.[32,33,34] Many of these children were already symptomatic and might benefit from a gluten-free diet.[32,34]. These findings may

challenge the decisive role of histology altogether, suggesting that the diagnosis of celiac disease should be based more on overall assessment of the clinical, serological, and histological information available.[35]. Altogether, the decision whether to proceed to biopsy in a child with low positive TGA and, on the other hand, whether to initiate a treatment trial or only close follow-up in cases with normal or inconclusive histology needs careful consideration and a decision taken jointly with the family.

The main strength of the present study was the use of well-defined prospective cohorts with excellent participation rates. We were also able to utilize sophisticated diagnostic methods systemically in two countries with disparate healthcare systems and patient characteristics. As a limitation, we were not able to calculate how often re-cutting of inappropriate biopsies was actually needed. In addition, in contrast to the 2012 ESPGHAN guidelines, symptoms were not used as a diagnostic criterion as we aimed to include heterogeneous phenotypes. In light of the mounting, although somewhat contradictory, evidence of the accuracy of serology in asymptomatic children,[15,36,37,38] the updated criteria allow a non-biopsy approach in this subgroup, too.[9] We had too few cases for a reliable appraisal of this issue, but the results nonetheless give further support to the conception that clinical presentation is of limited importance in setting the diagnostics. The use of only one TGA test could be seen as another limitation, but we did not aim to compare the performance of different assays as this has already been done.[10,25] It must also be noted that our results are restricted to pediatric population and further validation of the non-biopsy approach in adults is still required.

Conclusions

By applying histomorphometric tools on correctly oriented biopsy samples we demonstrated the high accuracy of the ESPGHAN non-biopsy criteria in different settings. Owing to the challenges with the

histological analysis, serology-based diagnostics with proven accuracy could be considered an easier and even more reliable approach to establish a celiac disease diagnosis in clinical routine.

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Figure legends

Figure 1. Flowchart of the study. CD: celiac disease; TGA; transglutaminase 2 antibodies; ULN: upper limit of normal; EmA, endomysial antibodies; HLA: human leukocyte antigen

Table 1. Children with positive IgA class transglutaminase 2 antibodies (TGA) of whose celiac disease (CD) diagnosis changed after precise morphometric evaluation of the duodenal biopsies

No.	Sex	Age, years	Symptoms	Centre	TGA, U/ml	EmA, titer	Original report	CD3+ IELs	VH:CrD[a]	New outcome	HLA DQ2/8
TGA < 10 ULN											
1	F	2.6	Asymptomatic	Romania	21	1:50	No CD	30	1.0	CD	Positive
2	M	5.3	Growth retardation	Finland	11	1:5	No CD	18	1.7	CD	Positive
3	M	3.8	Rash	Finland	14	1:50	No CD	22	1.6	CD	Positive
4	F	4.3	Abdominal pain	Finland	11	1:50	No CD	18	1.3	CD	Positive
5	F	5.2	Rash	Finland	23	1:5	No CD	44	0.3	CD	Positive
6	F	14.4	Abdominal pain	Finland	28	1:100	No CD	18	1.3	CD	Positive
7	F	7.2	Constipation, rash	Finland	32	1:200	No CD	30	1.4	CD	Positive
8	F	7.1	Asymptomatic	Finland	34	1:100	No CD	17	0.3	CD	Positive
9	F	4.2	Abdominal pain, anemia	Finland	64	1:200	No CD	29	0.5	CD	Positive
TGA ≥ 10 ULN											
10	M	3.7	Growth retardation	Finland	>120	1:4000	No CD	50	1.3	CD	Positive

[a]VH:CrD >2.0 is considered normal. EmA, endomysial antibodies; HLA, human leucocyte antigen, IEL, intraepithelial lymphocytes, cells/100 enterocytes; ULN, upper limit of normal; VH:CrD, villous height crypt depth ratio

Table 2. Characteristics in 163 consecutive children with either high or low positive IgA class transglutaminase 2 antibodies (TG2ab)

	TG2ab		
	≥10x ULN n= 88	<10x ULN n= 75	P value
Age, median (quartiles), yr	5.8 (4.5, 11.2)	7.2 (4.4, 11.6)	0.470
Girls, %	69.3	72.0	0.733
Celiac disease in family, %	34.1	52.0	**0.025**
Main clinical presentation, %			
Gastrointestinal	75.6	71.6	0.597
Extraintestinal[a]	17.4	17.6	0.961
Screen-detected[b]	7.0	10.8	0.413
Gastrointestinal symptoms, %			
Abdominal pain	52.9	57.9	0.574
Diarrhea	31.0	30.0	0.655
Nausea or vomiting	3.5	1.4	0.480
Constipation	14.9	11.4	0.532
Anemia, %	23.9	10.7	**0.053**
Concomitant conditions, %			
Celiac disease-associated[c]	3.4	9.3	0.189
Other chronic[d]	28.4	33.3	0.502

[a]E.g. poor growth, transaminasemia, rash, arthralgia; [b]Celiac disease in the family, previous type 1 diabetes; [c]Type 1 diabetes, autoimmune thyroidal disease; [d]E.g. asthma, epilepsy, food allergy. ULN, upper limit of normal

Table 3. Selected laboratory values and body mass index in 163 consecutive children with either high (≥10x ULN) or low (<10x ULN) positive IgA class transglutaminase 2 antibodies (TG2-ab)

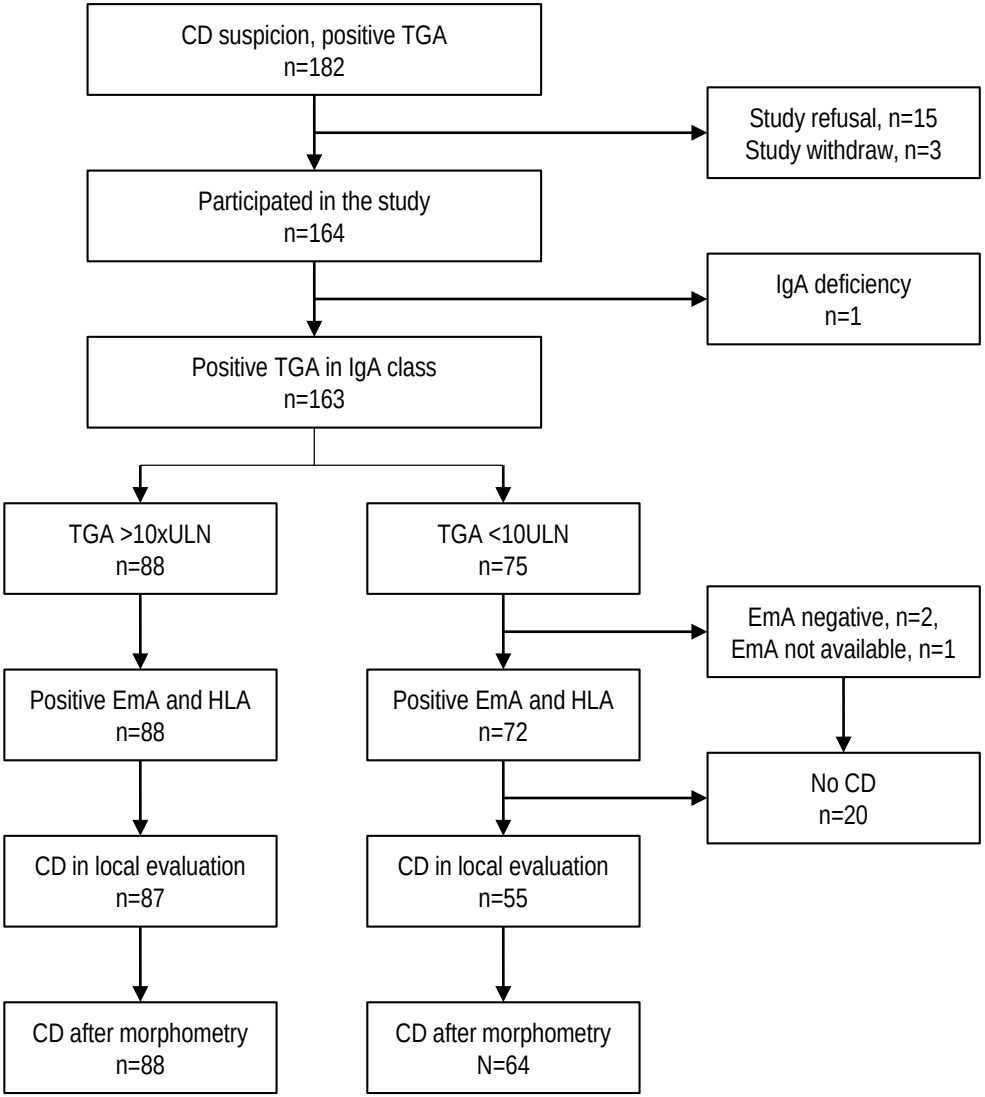
	High TG2ab n= 88	Low TG2ab n= 75	P value
	Median (quartiles)	Median (quartiles)	
Endomysium antibodies, titre	1:1000 (1:500, 1:4000)	1:100 (1:50, 1:200)	**<0.001**
Hemoglobin, g/l	122 (110, 131)	125 (117, 132)	0.114
Mean corpuscular volume, fl	79 (74, 82)	81 (76, 84)	**0.011**
Ferritin[a], µg/l	12 (6, 23)	22 (14, 37)	**0.001**
Alanine aminotransferase[b], U/l	23 (18, 33)	17 (13, 22)	**0.002**
Albumin[c], g/l	39 (36, 42)	37 (36, 41)	0.290
Body mass index[d], SDS	-0.9 (-1.8, 0.1)	-0.1 (-0.9, 0.4)	0.068

Data was available in >90% of the cases except in [a]50,[b]57, [c]44 and [d]67 cases.
ULN, upper limit of normal; HLA, human leucocyte antigen; SDS, standard deviation score

Table 4. Characteristics of 88 consecutive children from Romania and Finland fulfilling the triple positivity for celiac disease

	Romania n=23	Finland n=65	P value
Age, median (quartiles), yr	5.1 (3.0, 11.8)	6.7 (4.9, 11.6)	0.188
Girls, %	56.5	73.9	0.187
Celiac disease in family, %	27.3	36.5	0.602
Main clinical presentation, %			
Gastrointestinal	72.7	76.6	0.889
Extraintestinal	13.6	18.8	0.750
Screen-detected	13.6	4.7	0.181
Gastrointestinal symptoms, %			
Abdominal pain	39.1	57.8	0.225
Diarrhea	39.1	28.1	0.537
Nausea or vomiting	0	4.7	0.471
Constipation	8.7	17.1	0.502
Anemia, %	13.0	27.7	0.363
BMI-SDS, median (quartiles)	-1.9 (-2.5, -0.3)	-0.4 (-1.1, 0.5)	**<0.001**
Concomitant conditions, %			
Celiac disease-associated	8.7	1.53	0.166
Other chronic	8.7	35.4	**0.016**
Laboratory values, median (quartiles)			
Hemoglobin, g/l	125 (119-130)	119 (109-133)	0.344
Mean corpuscular volume, fl	82 (78-84)	78 (74-81)	**0.022**
Ferritin[a], µg/l	31 (6-49)	8 (6-16)	**0.003**
Alanine aminotransferase[b], U/l	37 (24-46)	20 (15-28)	**0.001**
Albumin[c], g/l	42 (37-45)	38 (37-40)	0.089

Data was available in >90% of the cases except in [a]51 cases, [b]66 cases and [c]42 cases
BMI-SDS, body mass index-standard deviation score


CD suspicion, positive TGA
n=182
Study refusal, n=15
Study withdraw, n=3
Participated in the study
n=164
IgA deficiency
n=1
Positive TGA in IgA class
n=163
TGA >10xULN
n=88
TGA <10ULN
n=75
EmA negative, n=2,
EmA not available, n=1
Positive EmA and HLA
n=88
Positive EmA and HLA
n=72
No CD
n=20
CD in local evaluation
n=87
CD in local evaluation
n=55
CD after morphometry
n=88
CD after morphometry
N=64