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Antibodies against deamidated gliadin peptide (DGP) identifies adult celiac disease patients negative for antibodies against endomysium and tissue transglutaminase (tTG)

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Running title: DGP in celiac disease

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Abstract

Aim: To evaluate diagnostic utility of antibodies against deamidated gliadin peptide (DGP).

Methods: Sera from 176 adults, referred for endoscopy without previous analysis of antibodies against tissue transglutaminase (tTG) or endomysium (EmA), were retrospectively analyzed by ELISAs detecting IgA/IgG-DGP or a mixture of DGP and tTG, and compared with IgA-tTG and EmA. Seventy-nine individuals were diagnosed with celiac disease (CD).

Results: Receiver operating characteristic (ROC) analyses verified the manufacturers' cut-off limits except for IgA/IgG-DGP/tTG. In sera without IgA deficiency the sensitivity was higher for IgA/IgG-DGP (0.85-0.87) compared to IgA-tTg (0.76) and EmA (0.61). All tests showed high specificity (0.95-1.00). Eighteen CD-sera were negative regarding IgA-tTG, nine of which were positive for IgA/IgG-DGP. Sera from CD-patients >70 years were more often negative for IgA-tTG (50%) and IgA/IgG-DGP (36%) than younger patients (15% and 8% respectively) ($p<0.01$). Three of the four IgA-deficient patients were positive in the IgA/IgG-DGP assay.

Conclusions: In this study of patients unselected regarding IgA-tTg/EmA, thus unbiased in this respect, IgA/IgG-DGP identified adult celiac disease patients negative for antibodies against endomysium and tissue transglutaminase. Serology is often negative in elderly patients with CD, a small bowel biopsy should therefore generously be performed before CD is excluded.

Key-words: adult celiac disease, deamidated gliadin peptide, diagnostic utility, tissue transglutaminase, antibodies.

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Introduction

Celiac disease (CD) is an immune-mediated enteropathy induced by dietary gluten. In genetically predisposed individuals, toxic gliadin peptides lead to inflammation of the small bowel mucosa with a subsequent risk for malabsorption and a wide range of disease manifestations [1, 2].

Gliadin peptides are deamidated by mucosal tissue transglutaminase (tTG) resulting in increased affinity for the CD-associated HLA DQ2 or DQ8 [3, 4]. In genetically predisposed individuals, an immune response can be elicited and antibodies produced against epitopes of gliadin and tTG [5, 6]. The fact that 'non-classical symptoms' and even absence of symptoms is frequent, demand reliable serological disease markers to select appropriate candidates for intestinal biopsy. It is widely accepted that antibodies of IgA-isotype against endomysium (EmA) and tTG have higher diagnostic accuracy than antibodies against gliadin (AGA). By using native gliadin as antigen source a wide range of antibodies against a mix of non-deamidated gliadin proteins are detected. However, by the use of deamidated instead of native gliadin the diagnostic specificity is strongly enhanced [7-11].

For assays detecting IgA-class antibodies against human recombinant tTG, a meta-analysis reported a sensitivity of 98.1% (95% CI: 90.1-99.7%) and a specificity of 98.0% (95% CI: 95.8-99.1%) in adult CD. For EmA sensitivity and specificity were reported to be 97% (95% CI: 95.7-98.5%) and 99.6% (95% CI: 98.8-99.9%) respectively [12]. However, it must be kept in mind that most studies report falsely high sensitivity due to biased study populations selected by positive serology [13]. Age-related differences of serology-responses in adults have been noticed and lower sensitivity has been reported for EmA in higher age-groups [14, 15]. Generally, antibodies of IgA isotype are used as seromarkers in CD, but in case of IgA-deficiency,

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IgG-specific assays are useful [16]. The prevalence of IgA-deficiency in the general population is about 1:500 and these individuals are at 10-fold increased risk for CD [17]. Therefore, it would be valuable to obtain a reliable diagnostic test identifying antibodies of both IgA and IgG isotype.

A meta-analysis comparing the performance of the IgA-DGP tests with IgA-tTG tests concluded that the IgA-tTG is preferable [18]. However, a major limitation was that the study populations in most studies were selected due to positive tTG-serology. The aim of this retrospective study was to evaluate assays simultaneously detecting serum IgA and IgG antibodies against either a mixture of purified human tTG and DGP, or to DGP alone in adults referred for endoscopy due to dyspepsia or suspected malabsorption. The patients had not previously been tested for antibodies against tTG and or EmA and thus unbiased in this respect. Further, we wished to compare these assays with IgA antibodies against tTG and endomysium respectively.

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Materials and methods

Patients

From a local serum bank, we identified samples from 176 patients (96 women and 80 men; median age 52, range 18-83) who had undergone endoscopy and small bowel biopsy without previous serologic testing for anti-tTG or EmA avoiding ascertainment bias. The patients were investigated between 1984 and 2001 and had been referred due to suspected malabsorption or dyspepsia as pain, bloating or loose stools. Blood sampling was performed at the time of endoscopy. The sera have been stored at -≤20°C. Based on histological findings 79 patients were diagnosed with celiac disease. Biopsies from 97 subjects did not fulfill histological criteria for celiac disease and these

patients have until 2009 not been diagnosed with the disease. No patient was on gluten free diet at sampling.

Small bowel biopsy

One biopsy was obtained from the proximal small bowel at the place of ligamentum Treitz using a Watson capsule (only before 1992). At endoscopy four biopsies were taken from the descending part of the duodenum during endoscopic investigation. The biopsies were re-evaluated (by SI) using the Marsh-Oberhober classification and CD3-staining [19]. More than 20 intraepithelial lymphocytes (IEL) per 100 enterocytes were classified as Marsh I. The re-evaluation was performed without access to the serological outcome reducing risk for diagnostic bias. Histological changes corresponding to at least Marsh grade 3a was required to fulfill the criteria for celiac disease in all but one patient with dermatitis herpetiformis who had Marsh grade 1 initially.

Antibody analyses

Commercially available enzyme-linked immunosorbents assays (ELISAs) were used according to the manufacturers' instructions. Optimal cut-off values were determined by receiver operating characteristic (ROC) analyses. The following ELISAa were performed:

1. IgA- and IgG-class antibodies against a combination of DGP and tTG (Quanta Lite ™ h-tTG/DGP Screen, INOVA Diagnostics, San Diego, CA, USA). Manufacturer's cut-off: 20 AU/mL. ROC-corrected cut-off: 35 AU/mL.
2. IgA- and IgG-class antibodies against DGP (Quanta Lite ™ Celiac DGP Screen, INOVA Diagnostics, San Diego, CA, USA). Cut-off: 20 AU/mL.

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3. IgA-antibodies against human recombinant tTG (Celikey, Phadia, Freiburg, Germany). Cut-off: 5 U/mL

Antibodies against endomysium were analysed by indirect immunofluorescence (IF) microscopy using fixed sections of monkey esophagus (Bio systems S.A, Barcelona, Spain), and fluorescein-isothiocyanate conjugated rabbit anti-human IgA antibodies (DAKO A/S, Glostrup, Denmark). Visible reaction at a serum dilution of 1:≥5 was considered positive.

Serum IgA levels were determined by a routine turbidimetric method.

Statistical analysis

The sensitivity and specificity with 95% confidence interval as well as accuracy and diagnostic odds ratio (DOR) were calculated. ROC analysis was performed to identify optimal cut-off values. Comparisons between groups were performed by Chi^2-test, McNemar's and Mann-Whitney's test.

Ethical considerations

The study was approved by the regional ethics committee, Linköping.

Results

Small bowel biopsy analyses

Seventy-nine patients (Figure 1) were diagnosed with celiac disease based on histological findings, four of which were IgA-deficient.

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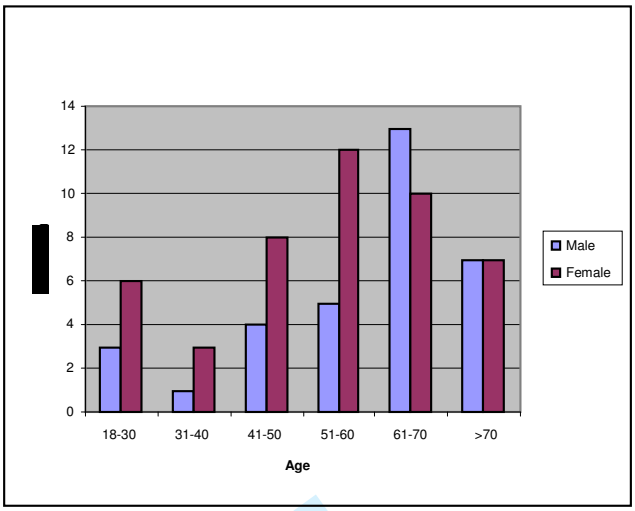

Figure 1 Age and gender in the 79 patients with celiac disease

Antibody analyses

Sera from 97 individuals who did not fulfill histological criteria for CD

None of the individuals in this group was IgA deficient. The diagnostic specificities of the tests (with ROC-verified cut-offs) were 0.96 – 1.00 (Table 1). Positive serology was found in 11 (11%) out of these 97 sera, all of which were weakly positive. Five sera (5%) were positive regarding IgA antibodies against human recombinant tTG in the Phadia assay, but negative in the other tests, including the Inova test containing human purified tTG. Another four sera (4%) were positive in the Inova DGP test, and only one of these was also positive in the combined tTG/DGP assay. Another two sera were positive in the Inova tTG/DGP test alone.

Positive serology was more frequent among men (19%) as compared to women (4%) without CD ($p<0.05$). Histological changes of Marsh grade 0 and I did not differ in those with positive serology (Table 2).

None of these 97 individuals have until now (after 8-25 years) been diagnosed with CD.

None of these 97 sera was EmA-positive.

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Using the manufacturer ´s cut-off (20 AU/mL) for the tTG/DGP-test the specificity was reduced to 0.80.

Table 1

Sensitivity, specificity, accuracy and diagnostic odds ratio (DOR) with 95% confidence intervals in patients without IgA-deficiency

	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy	DOR (95% CI)
IgA/IgG-DGP	20 AU/ml	0.87 (0.81-0.95)	0.96 (0.92-1.00)	0.92	171 (50-577)
IgA/IgG-tTG/DGP	Recommended 20 AU/ml	0.91 (0.85-0.97)	0.80 (0.72-0.88)	0.85	40 (16-101)
IgA/IgG-tTG/DGP	ROC-corrected 35 AU/ml	0.85 (0.77-0.93)	0.98 (0.95-1.00)	0.92	278 (59-1288)
IgA-tTG	5 U/ml	0.76 (0.66-0.86)	0.95 (0.91-0.99)	0.87	58 (21-166)
IgA-EmA	serum-dilution 1/5	0.61 (0.50-0.72)	1.00 (1.00-1.00)	0.83	307 (18-5141)

Table 2

Positive serology in patients not fulfilling histological criteria for CD

Patient	Gender	Age	IgA/G-anti-tTG/DGP <35 AU/mL	IgA/G-anti-DGP <20 AU/mL	IgA-anti-tTG <5 U/mL	IgA-EmA	Marsh
1	M	55	51	41	neg	neg	M1
2	F	50	neg	33	neg	neg	M1
3	M	29	neg	32	neg	neg	M0
4	M	53	neg	25	neg	neg	M0
5	M	59	55	neg	neg	neg	M1
6	M	18	40	neg	neg	neg	M1
7	M	25	neg	neg	7.3	neg	M1
8	M	76	neg	neg	6.7	neg	M1
9	M	32	neg	neg	5.8	neg	M0
10	M	29	neg	neg	5.5	neg	M1
11	F	49	neg	neg	5.3	neg	M0

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Sera from the 79 individuals fulfilling histological criteria for CD

Four individuals (5%) in this group had IgA deficiency.

Fifty-seven (76%) of 75 sera without IgA deficiency were positive in the Phadia IgA-tTG-assay and 46 (61%) were EmA positive. Sixty-five sera without IgA deficiency (87%) were positive in the Inova IgA/IgG-DGP.

When using the manufacturer's recommended cut off (20 AU/mL) in the IgA/IgG-DGP/tTG-test the sensitivity was 0.91 compared to 0.85 with ROC-corrected cut off (35 AU/mL). Sensitivities with 95% CI are shown in Table 1.

Eighteen of the 75 (24%) sera without IgA deficiency were negative in the tTG-Phadia assay. Of these one (6%) was weakly IgA-EmA positive, whereas nine (50%) were positive in the IgA/IgG-DGP and eight (47%) in the IgA/IgG-DGP/tTG-test (ROC-corrected cut-off 35 AU/mL). Using McNemar's test there were differences between the Inova IgA/IgG-DGP and Phadia IgA-tTG ($p<0.05$) and also EmA ($p<0.001$).

The IgA/IgG-DGP positive/IgA-tTG negative CD-patients did not differ histologically from the IgA-tTG-positive CD-cases (Table 3).

Table 3

Characteristics of the nine patients (without IgA-deficiency) negative for IgA-tTG but positive IgA/IgG-DGP.

Patient	Gender	Age	IgA/IgG-tTG/DGP (<35 AU/mL)	IgA/IgG-DGP (<20 U/mL)	IgA-tTG (<5 U/mL)	IgA-EmA serumdilution 1/5	Marsh
1	F	41	116	71	3.5	pos	M3a
2	F	55	138	94	3.0	neg	M3c
3	F	81	29	20	0	neg	M3a
4	F	54	112	92	3.0	neg	M1*
5	F	78	135	116	0	neg	M3c
6	M	66	70	62	4.4	neg	M3a
7	M	50	48	25	4.6	neg	M3a
8	M	82	90	67	1	neg	M3c
9	M	50	78	37	2	neg	M3a

* Dermatitis herpetiformis

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CD-sera from patients > 70 years of age were more often negative for both IgA-tTG (7/14, 50%) and IgA/IgG-DGP (5/14, 36%) than in patients ≤70 years (11/61, 18% and 5/61, 8% respectively) (p<0.01). This finding was even more pronounced using EmA (fig 2).

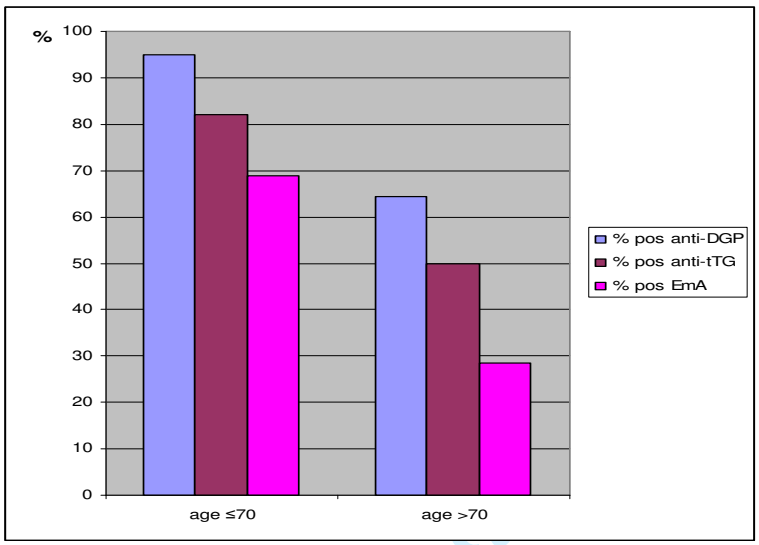

Figure 2 Proportion of positive serology in CD patients without IgA deficiency > 70 years (n=14) and ≤70 years of age (n=61).

The four IgA deficient sera were negative regarding IgA-tTG, whereas three of them were positive in the IgA/IgG-DGP and the IgA/IgG-DGP/tTG-test.

Discussion

This study shows that the INOVA Diagnostics assays, that simultaneously detect IgA- and IgG-class antibodies against a mixture of tTG and DGP or DGP alone, are both highly sensitive and specific for adult CD. This is in line with previous reports on IgA-DGP, IgG-DGP and IgA/IgG-DGP in adult CD [7, 10, 11, 20-23] as well as childhood CD [9, 24, 25].

Somewhat unexpectedly, we found that antibodies against DGP was the only positive seromarker among a substantial number of patients with biopsy-verified CD and negative serology for the traditional markers IgA-tTG and EmA without being IgA

deficient. These patients did not seem to differ clinically or histologically from the IgA-tTG and/or IgA-EmA positive CD patients, but further prospective studies are warranted to elucidate distinct clinical baseline characteristics as well as differences regarding disease course and outcome.

Another important finding in this study is that many of the elderly CD patients (> 70 years of age) were antibody-negative without clear differences between the different tests. Previous reports have suggested that CD serology may perform less well in higher age groups [14, 15]. This is clearly confirmed in our study and underlines the importance of performing small bowel biopsy even in seronegative elderly patients with clinically suspected CD. Importantly, negative CD serology may thus be misleading, especially as the elderly often have vague symptoms [26]. High negative predictive values are often reported in studies based on younger populations with high prevalence of CD and the use of high-sensitivity tests. However, these CD tests are seldom validated in the highest age groups. Thus, findings achieved from younger CD patients, may be erroneously extrapolated to the elderly.

The general view has long been that EmA and anti-tTG tests are highly correlated and equally suited for diagnostic purposes due to their high diagnostic specificity as well as sensitivity [12]. However, although EmA is a highly disease-specific CD marker, it is not sensitive enough for screening purposes, as shown here and by others [14, 21, 27, 28]. As compared to young adults, a lower EMA-prevalence has been reported in higher age groups [14, 29]. Since half of our study population was ≥ 50 years of age, this could be an explanation to our finding of low sensitivity of EmA. Even though celiac-disease associated antibodies often recognize epitopes common to both tTG and endomysium, it is known that there are also fractions of antibodies recognizing epitopes of deamidated gliadin and transglutaminase 2, but not endomysium [30]. Another explanation to our

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finding of low sensitivity for EmA may be that our patients, in contrast to most other studies, were not selected due to the presence of antibodies against endomysium and/or tTG, thus avoiding ascertainment bias. Our finding that a substantial proportion of the anti-tTG/EmA negative CD sera were positive in the DGP assays implies that a DGP-assay should be included in serological testing for CD. Whether the divergent results between the different ELISAs depend on different antigen sources or is due to other technical reasons has not been possible to evaluate in this study. This important issue has previously been highlighted in studies comparing different tTG, EmA and DGP assays [31-33].

We could verify the manufacturers' recommended cut-off level for both IgA-tTg (Phadia) and IgA/IgG-DGP (Inova). However, after ROC analysis, the cut-off level for the combined IgA/IgG-tTG/DGP test was found to be 35AU/mL instead of recommended 20AU/mL. The reason for this is unclear and, due to the small sample size in the present study, the ROC-corrected cut-off level should be interpreted with some caution and thus not be generally applied without confirmation. Further studies on larger materials are needed to elucidate this matter.

With the manufacturer's cut-off level, the sensitivity of the combined DGP/tTG assay (in patients without IgA deficiency) was 91%, whereas the diagnostic specificity was only 80%, which is unacceptably low, and even lower than previously reported (89%) for specificity in childhood CD [24]. Increasing the cut-off level to 35 AU/mL according to ROC analysis, increased the specificity to 98% which is in line with the IgA/IgG-DGP and IgA-tTG assays.

Although assays detecting antibodies to human recombinant tTG are considered highly sensitive in CD [12], the specificity has been questioned since such antibodies have also been described in patients with end-stage heart failure, myocardial infarction, and primary

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biliary cirrhosis without evidence of concomitant CD [34-36]. . However, our five patients with positive IgA-tTG serology but lacking histological findings of CD were all weakly positive in the grey-zone. The IgA/IgG-tTG/DGP assay does not seem to add any advantage to the assay detecting antibodies against DGP alone.

None of the patients lacking histological signs of CD in the duodenal biopsy has yet been diagnosed with CD. This strengthens the diagnostic value of the DGP assays with their 92% accuracys compared to 87% accuracy for the routine tTG test Celikey. Also, the diagnostic odds ratios (DOR) that discriminate the power of diagnostic tests [37] showed higher values for IgA/IgG-DGP (171) than for IgA-tTG (58).

One possibility is that patients with localized inflammation may have been misdiagnosed as normal. However, multiple biopsies taken from the duodenal mucosa at endoscopy is strongly correlated with biopsies from jejunum obtained by capsule or endoscope [38, 39].

A weakness with our study is that only a few of the patients lacking histological signs of CD 10-25 years ago, have since been re-biopsied or serologically retested. They have been followed by their general practitioner but have not had symptoms justifying a new investigation/biopsy during these years.

A retrospective study design has limitations, and a prospective study with a larger sample size would have been preferable. However, nowadays almost all patients referred for small bowel biopsy have been tested regarding CD serology and thus flawed by ascertainment bias.

In conclusion, antibodies against DGP seem to identify a substantial number of true CD cases with negative serology regarding the traditional CD markers IgA-tTG and IgA-EmA, even in the absence of IgA deficiency. In our small study, 12% of the true CD cases could only be serologically identified by the DGP tests. Hence, the anti-DGP antibody test appears to be an important diagnostic tool in adult celiac disease. ROC

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analysis indicated that cut-off limits assessed at the local diagnostic laboratory are valuable, although our material was not large enough to substantiate this notion.

Negative CD serologi, including IgA/IgG-DGP, was found to be frequent among the elderly patients with CD. We therefore recommend small bowel biopsy in elderly patients with negative serology before CD is excluded. Furthermore, the low sensitivity of EmA in a serologically unbiased population underlines that it is not a first line test, despite its excellent specificity.

Conflict statement

None of the authors has any financial or other relationship to any of the manufacturers of the used assays that might lead to a conflict of interest.

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