RESEARCH ARTICLE

Hemolysis and IgA-antibodies against tissue transglutaminase: When are antibody test results no longer reliable?

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Background: Antibodies against tissue transglutaminase (TTG) of isotype IgA (IgA-aTTG) represent reliable diagnostic markers to confirm or exclude celiac disease (CD). Hemolysis (HL) is an important pre-analytical factor. HL can be quantified as HL index (HI) correlating with the concentration of free hemoglobin. TTG is abundant in erythrocytes and released upon HL. In immunoassays, the released TTG may interfere with binding of IgA-aTTG to the coated TTG.

Methods: We selected 17 HL-free sera from children with biopsy-confirmed CD: 7 with low-positive (1-5 multiples of upper limit of normal [$\times$ ULN]), 5 with intermediate (5-10 $\times$ ULN) and 5 with high IgA-aTTG (10-15 $\times$ ULN). Sera were spiked with hemolysates resulting in HIs ranging from 12.5 to 800 (12.5-800 mg/dL free hemoglobin).

Results: IgA-aTTG values were significantly decreased ($>10\%$) after addition of hemolysates even if HL was invisible (HI <50). This effect is diagnosis-relevant if IgA-aTTG values are measured just below the cut-offs: (i) 0.4-1 $\times$ ULN at HI ≥ 25 (CD not excludable) and (ii) 8.5-10 $\times$ ULN at HI ≥ 200 (diagnosis of CD without biopsy not possible). Antibodies against deamidated gliadin were not influenced by HL.

Conclusions: IgA-aTTG results in sera with HI ≥ 25 can yield inconclusive results. Therefore, those antibody results should be assessed only under consideration of the HI.

KEYWORDS

autoantibodies, celiac disease, deamidated gliadin, hemolysis index, pre-analytics

1 | INTRODUCTION

Celiac disease (CD) is an autoimmune-mediated enteropathy with tissue transglutaminase (TTG) as autoantigen¹ triggered by an abnormal immune response to wheat gluten and related cereal peptides in genetically predisposed persons.² The diagnosis is based on the

assessment of highly variable clinical symptoms, antibody measurements, histological evaluation of intestinal biopsy samples, and response to gluten-free diet.³ Measurement of IgA-antibodies against tissue transglutaminase (IgA-aTTG) currently represents the most sensitive and specific antibody test in the diagnosis of CD. Diagnostic sensitivity of IgA-aTTG assays after exclusion of patients with IgA-deficiency reaches almost 100%.⁴ Assays for measuring the autoantibodies apply the recombinant TTG coated to plastic surfaces⁵ or magnetic beads⁶ to capture autoantibodies in sera.

Hemolysis (HL) is one of the most relevant pre-analytical events influencing the validity of results of laboratory diagnostics. Traditionally,

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Abbreviations: CD, celiac disease; CI, confidence interval; HI, hemolysis index; HL, hemolysis; IgA-aTTG, IgA antibodies against TTG; IgG-aDGL, antibodies against deamidated gliadin; TTG, tissue transglutaminase.

the identification of hemolytic samples has been performed by visual inspection of the color of serum or plasma. However, free hemoglobin below 50 mg/dL is not visible and concentrations between 50 and 130 mg/dL are difficult to identify.⁷ Since a couple of years, free hemoglobin is routinely quantified photometrically in parallel to most biochemical assays and a HL index (HI; 1 index point = ~1 mg free hemoglobin per 1 dL) is calculated. TTG is abundant in erythrocytes^{8,9} and released upon disruption of the membrane of red blood cells into the serum. The released erythrocytic TTG could interfere with binding of IgA-aTTG to the coated recombinant TTG. Therefore, a recent report¹⁰ recommended that aTTG should not be determined in samples with visible HL. Furthermore, the authors showed that HL did not affect the detection of IgG-antibodies against deamidated gliadin (IgG-aDGL). The current study aims to support the interpretation of IgA-aTTG measurement in samples affected by HL.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The use of the serum samples for scientific purpose was approved by the ethical committee of the University of Leipzig (ethical permit number: 116-2009-25052009). Informed consent was obtained from all individual participants included in the study. For children, we obtained written informed consent from the next of kin, caretakers, or guardians, respectively. The consent procedures and the informed consent documents were approved by the ethic committee. Blood for the trial was withdrawn during routinely performed blood removal.

2.2 | Samples

To investigate the effect of HL on assay of IgA-aTTG at different concentrations, we selected 17 serum samples from children and adolescents with biopsy-confirmed CD with HI <5. The concentration of IgA-aTTG in these samples was known in advance from prior measurements with an assay of EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany). The concentration of IgA-aTTG (expressed as multiples of upper limit of normal [$\times$ ULN] applying a cut-off of 20 U/mL) was low ($1\text{--}5 \times$ ULN) in 7 serum samples (group 1), intermediate ($5\text{--}10 \times$ ULN) in 5 samples (group 2), and high ($10\text{--}15 \times$ ULN) in 5 samples (group 3). Furthermore, we investigated 2 sera, which exhibited false-positive values for IgA-aTTG (CD clinically and histologically excluded). All serum samples were collected around the time of duodenal biopsy under normal gluten-containing diet and aliquots were stored until measurement at -20°C . The data of these patients were already included in recent publications.^{11,12}

2.3 | Spiking of serum samples with hemoglobin

To simulate different levels of HL, we generated artificial hemolysates. For that purpose, we isolated the cellular compartment from EDTA-blood by centrifugation and washed the cell pellet 3 times with 0.9% NaCl. Afterward, the cell pellet was resuspended in distilled water

and frozen over night at -20°C . After thawing and rough shaking, the hemolysate was centrifuged again and the supernatant was used for further experiments. In the supernatant, the HI was determined photometrically on a Roche Cobas 8000 Modular Analyzer (Basel, Switzerland) at wavelengths of 570/600 nm. One index point is defined as 1 mg free hemoglobin per 1 dL. Each serum sample was spiked with a dilution series of the hemolysate supernatant to obtain a final HI of 12.5, 25, 50, 100, 200, 400, and 800 (12.5–800 mg free hemoglobin per dL). For control, each serum sample was also spiked with a serum containing IgA-aTTG below the measurement range and free hemoglobin of 7 mg/dL (non-visible). To feign the time gap between withdrawal of blood, transport to laboratory, sample processing, storage time, and measurement of autoantibodies, spiked samples were incubated for 2 hours at room temperature prior to the measurement. To ensure that the calculated HI is present in the spiked samples, we performed control measurements of HI in selected samples after spiking and confirmed the hemoglobin concentration (data not shown).

2.4 | Antibody assays

IgA-aTTG were measured by ELISA with assay A (cut-off ≥ 20 U/mL; EUROIMMUN Medizinische Labordiagnostika AG) and with assay B (Inova Diagnostics, cut-off ≥ 4 U/mL; Quanta Lite®, San Diego, USA) following the manufacturer's instructions. For measuring IgG-aDGL, we applied an ELISA kit of EUROIMMUN Medizinische Labordiagnostika AG (cut-off ≥ 25).

A decrease of the IgA-aTTG concentration by at least 10% (twofold of the mean inter-assay variation) was considered not to be caused by error of the ELISA tests but by HL.

2.5 | Hemolytic samples tested for IgA-aTTG in our children's hospital

To estimate how often the IgA-aTTG assay may be influenced by HL, we searched the LabCentre data base with anonymized patient data (i-SOLUTIONS Health GmbH, Mannheim, Germany) of our clinical routine lab for pediatric cases which were tested simultaneously for IgA-aTTG and HI between January 2015 and December 2016 in our laboratory (baseline and follow-up).

3 | RESULTS

3.1 | Hemolysis influences the measurement of IgA-aTTG

We observed a significant reduction of the IgA-aTTG concentration after spiking of serum samples with hemolysate aliquots. The degree of reduction depended on IgA-aTTG concentrations before spiking and on the amount of the added hemolysate (Figure 1). For assay A, the critical HI threshold (decrease by at least 10%) was 25 for sera with IgA-aTTG concentrations between 1 and $5 \times$ ULN (group 1), 100 for sera with intermediate IgA-aTTG concentrations between 5 and $10 \times$ ULN (group 2), and 200 for sera with IgA-aTTG between 10 and

FIGURE 1 Decrease of IgA-antibodies against tissue transglutaminase (IgA-aTTG) concentration after spiking of celiac patients' sera with hemolysate. For testing IgA-aTTG, assay A (A-C) or assay B (D-F) was used. For better comparison, antibody concentrations were normalized to multiples of the upper limit of normal and expressed as fraction of the values after spiking with hemolysis-free serum (bold black line). 95% confidence intervals are depicted as dashed lines. The dotted grey lines represent a decrease by 10%. Sera with IgA-aTTG between 1 and $5 \times$ ULN are shown in A and D, with IgA-aTTG between 5 and $10 \times$ ULN in (B) and (E) and with IgA-aTTG between 10 and $15 \times$ ULN in (C) and (F)

$15 \times$ ULN (group 3). Applying assay B, the critical HI thresholds were 50, 100, and 200, respectively. The mean half-maximum inhibitory concentration (IC_{50}) of HI for group 1 and 2 was 290 and 620 using assay A as well as 484 and 800 applying assay B. For group 3, a maximum decrease of 25% was observed at an HI of 800 for both tests.

Notably, independent of added hemolysate level, none of the samples reached the threshold value of the lower measurement range. This implies that IgA-aTTG values less or equal to the lower measurement range are true negative and not caused by present HL.

FIGURE 2 Celiac patients' sera with IgA-antibodies against tissue transglutaminase (IgA-aTTG) concentrations between 1 and 5 $\times$ ULN lead to false-negative results after spiking with hemolysate. IgA-aTTG concentrations are expressed as multiples of the upper limit of normal ($\times$ ULN) for each serum for assays A and B. The bold dotted lines represent the cut-off. Individual patients are represented in A and B by the same line patterns and symbols

3.2 | Hemolysis and false-negative IgA-aTTG results

As depicted in Figure 2, there was one serum of group 1 with a low-positive IgA-aTTG concentration, which showed false-negativity in both tests even at a HI between 12.5 and 25. This low level of HL is not visible. Two low-positive sera of group 1 declined below the respective cut-offs when the HI was between 100 and 300, respectively. Notably, only 2 sera of group 2 decrease below the respective thresholds after induction of HL with an HI of 800 (data not shown). None of the sera of group 3 showed negativity even at an HI of 800. However, IgA-aTTG was decreased below the 10 $\times$ ULN in 3 of 5 sera at a HI of 200, and in all 5 sera at a HI of 800. None of the samples in group 1, 2, and 3 reached values below the measurement range after spiking with hemolysates.

3.3 | Hemolysis does not influence detection of IgG-aDGL

We also assayed IgG-aDGL in the spiked sera. Ten of 17 sera were positive for IgG-aDGL before spiking. The concentration of IgG-aDGL was not decreased by spiking in any of the samples, irrespective of the hemoglobin concentration (Figure 3).

3.4 | IgA-aTTG is not depressed in two false-positive sera by spiking with hemolysates

We also spiked 2 sera with the hemolysates, which were tested initially false-positive (CD clinically and histologically excluded) with assay A. Interestingly, both remained positive despite increasing HI applying assay A (Figure S1A). Only one of these sera was initially positive with assay B (Figure S1B). Also in assay B, spiking with hemolysates did not alter the concentration of IgA-aTTG in both sera.

FIGURE 3 Detection of IgG-antibodies against deamidated gliadins (IgG-aDGL) is not affected by hemolysis. Ten of the 17 sera of all 3 groups with positive IgA-aTTG were also positive for IgG-aDGL

3.5 | Frequency of hemolytic serum samples tested for IgA-aTTG

We found 1102 sera, which were tested simultaneously for IgA-aTTG and HI within 2 years in our clinical routine lab. In 317 of the cases (28.8%), the HI was ≥ 25 indicating a possible interference with assays of IgA-aTTG. Assuming that HL is not able to cause a reduction from positive IgA-aTTG concentrations ($>1 \times$ ULN) to levels below the measurement range (see above), only 41 of 317 sera remained with a HI ≥ 25 , in which the IgA-aTTG concentration might have been positive in the absence of HL. In 9 of these sera, the HI was ≥ 100 . In 3 cases, HL seemed to have an effect on IgA-aTTG detection because IgA-aTTG levels were between cut-off and the lower end of the detection range. The highest observed HI was 298.

FIGURE 4 Proposal for interpretation of IgA-antibodies against tissue transglutaminase (IgA-aTTG) concentration in the context of hemolysis (HL). The scheme is based on test results with assay A in Figure 1 (visualized by the filled black circles). In the white area, there is no diagnosis-relevant influence of HL on the concentration of IgA-aTTG. In the gray area, HL may cause – in dependence on the HL index (HI) – a decrease in the concentration of IgA-aTTG below the borders of $1 \times \text{ULN}$ or $10 \times \text{ULN}$, which are relevant for celiac disease diagnosis. Note that in both, the white and the gray areas, HL may reduce IgA-aTTG levels relevant for the assessment of the compliance of gluten-free diet. No sera with $\text{HI} > 300$ were found in our study

4 | DISCUSSION

The interference of HL is a main concern in laboratory medicine and could result in positive or negative bias in the measurement of various analytes.¹³ HL may occur already in vivo, eg, by increased fragility of erythrocytes due to inherited disorders, but is more common in the pre-analytic stage during or after blood withdrawal. Especially in the pediatric setting, HL is often caused by squeezing during blood withdrawal. Other causes of in vitro HL are mechanical destruction during sample transport or procession.¹⁴ Typical analytes, the measurement of which is affected by HL, are lactate dehydrogenase, potassium, and some tumor markers which are released from erythrocytes. In the modern laboratory, free hemoglobin can be determined in minimal sample volumes simultaneously with multiple analytes. This also offers the possibility to study the influence of HL on immunodetection of IgA-aTTG in more detail.

Tissue transglutaminase is abundant in red blood cells. Its content in erythrocytes exceeds that in other cell types.⁸ Red blood cell TTG¹⁵ is released after lysis of erythrocytes and competes with recombinant TTG coated to the plastic surface or to beads of the test kits. In a publication, which is unfortunately paid relatively little attention to by gastroenterologists and clinical chemists, it was recently shown that visible free hemoglobin interferes with measurements of IgA-aTTG.¹⁰ However, in clinical routine this source of diagnostic invalidity is not generally considered.

Here, we re-investigate the role of HL in detection of IgA-aTTG. We confirm the finding that the IgA-aTTG assay is affected by HL in

dependence on the concentration of free hemoglobin. In addition, we are able to show that the initial concentration of IgA-aTTG in the samples before spiking with hemolysates is important. Intermediate values ($5\text{--}10 \times \text{ULN}$) are less affected by the same amount of HL as those with low-positive values ($1\text{--}5 \times \text{ULN}$). Sera with high IgA-aTTG ($>10 \times \text{ULN}$) are not influenced if $\text{HI} < 200$. A very important finding is that erythrocytic TTG even may compete with coated TTG and decrease measured IgA-aTTG values when HL was invisible ($\text{HI} < 50$). This may cause false-negative results in sera with low-positive autoantibody concentrations. However, HL was not able to reduce IgA-aTTG from concentrations $>1 \times \text{ULN}$ to values below the measurement range in any of our samples, even at high HI (up to 800). In most samples of daily routine, the IgA-aTTG concentration is below the measurement range. Therefore, HL in these samples seems extremely unlikely to be relevant for diagnosis. Otherwise, samples with $\text{HI} \geq 25$ should always be considered affected by HL if the detectable IgA-aTTG concentration is negative but above the lower end of the measurement range, especially in case of positive IgG-aDGL – an antibody species which is not affected by erythrocytic TTG. The detection of IgG-aDGL is not influenced by HL since gliadin peptides are components of gluten which is found in wheat and related cereals. These peptides are not released during lysis of erythrocytes. Therefore, there is no inhibitory effect of HL on the binding of these antibodies to the presented antigen on plastic surfaces of different IgG-aDGL assays.

Hemolysis is a common pre-analytic error especially in pediatric diagnostics.¹⁴ An index of at least 25 was found in 28% of sera, which were tested for IgA-aTTG in our laboratory. However, most of these samples had IgA-aTTG concentrations below the measurement range, which are not suspicious of CD even in case of HL. Only in the remaining 3.7% of the sera with IgA-aTTG above the lower end of the measurement range, a diagnosis-relevant effect of HL has to be considered.

Arguelles-Grande et al¹⁰ stated that IgA-aTTG should generally not be measured in samples with visible HL. This is also suggested by most manufacturers of IgA-aTTG assays. We only partially agree with this. An effect of HL is only relevant for the diagnosis of CD if the concentration of antibodies is decreased from values above to values below the $1 \times \text{ULN}$ or $10 \times \text{ULN}$ border. Our data show that in samples with IgA-aTTG concentrations higher than $1 \times \text{ULN}$, the antibody concentration may be reduced down to $0.4 \times \text{ULN}$ by HL if the HI is above 25. This illustrates that CD cannot be excluded due to HL. Samples with $\text{IgA-aTTG} \geq 10 \times \text{ULN}$ are only affected in a diagnosis-relevant manner if the HI is ≥ 200 . Such high HI is only rarely observed in the daily routine (see Figure 4).

We recommend the assay of IgA-aTTG also in the presence of visible HL. In case IgA-aTTG concentrations between 8.5 and $10 \times \text{ULN}$ were measured, values $\geq 10 \times \text{ULN}$ should be taken into account if the HI is at least 200. The finding of an IgA-aTTG concentration $\geq 10 \times \text{ULN}$ is important for the pediatric gastroenterologist to enable a diagnosis of CD without biopsy.^{3,11,16} A graph helpful for interpretation of IgA-aTTG values measured in hemolytic samples is provided in Figure 4.

We observed marginal differences between assay A and B. These differences were found not only in sera with low-positive IgA-aTTG

concentrations but also in samples with higher values. The differences may be explained by varying analytical sensitivities of the 2 tests^{3,16} leading to a rearrangement of samples in our groups for different IgA-aTTG concentrations (see numbers in the right upper corner in Figure 1). Therefore, assay B seems less affected by HL in the group of samples which show low IgA-aTTG values. We speculate that false-positivity observed in 1 or 2 sera applying assay A or B, respectively, is due to binding of unspecific patient's antibodies to components of the blocking reagents used in the tests. A possible procedure to identify false-positive samples might be the addition of hemolyzed blood. Until now, we only had the chance to investigate these 2 samples and further evaluations of these findings are needed.

We assume that HL does not only influence the IgA but also the IgG-class of aTTG. IgG-aTTG is sometimes used for diagnostics in case of IgA-deficiency. The effect of HL on IgG-aTTG remains to be investigated.

In conclusion, laboratory physicians and gastroenterologists should interpret IgA-aTTG concentrations above the lower end of the measurement range with caution if the HI is above 25. Since IgA-aTTG values in samples with different HI are not comparable, the dietary compliance cannot be assessed accurately and dietary transgressions may be overlooked at the follow-up visit of the patient. The effect of HL is relevant for the diagnosis of CD if (i) IgA-aTTG decreased from values $>1 \times \text{ULN}$ down to $0.4 \times \text{ULN}$ in the presence of a HI ≥ 25 (CD mistakenly excluded) or if (ii) IgA-aTTG decreased from values $\geq 10 \times \text{ULN}$ down to $8.5 \times \text{ULN}$ in the presence of a HI at least 200 (diagnosis of CD without biopsy not possible).

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CONFLICTS OF INTEREST

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REFERENCES

- Dieterich W, Ehnis T, Bauer M, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med*. 1997;3:797-801.
- Green PHR, Cellier C. Celiac disease. *N Engl J Med*. 2007;357:1731-1743.
- Husby S, Koletzko S, Korponay-Szabo IR, et al. European society for pediatric gastroenterology. Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr*. 2012;54:136-160.
- van Meensel B, Hiele M, Hoffman I, et al. Diagnostic accuracy of ten second-generation (human) tissue transglutaminase antibody assays in celiac disease. *Clin Chem*. 2004;50:2125-2135.
- Sárdy M, Odenthal U, Karpati S, Paulsson M, Smyth N. Recombinant human tissue transglutaminase ELISA for the diagnosis of gluten-sensitive enteropathy. *Clin Chem*. 1999;45:2142-2149.
- Daves M, Cemin R, Perkmann V, et al. chemiluminescence IgA and IgG anti-tissue transglutaminase (tTG) antibodies serum assays for the screening of celiac disease. *J Immunol Methods*. 2016;429:57-59.
- Lippi G, Blankaert N, Bonini P, et al. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. *Clin Chem Lab Med*. 2008;46:764-772.
- Wolf J, Lachmann I, Wagner U, Osman AA, Mothes T. Quantification of human tissue transglutaminase by a luminescence sandwich enzyme-linked immunosorbent assay. *Anal Biochem*. 2011;419:153-160.
- Popp A, Jinga M, Jurcut C, et al. Fingertip rapid point-of-care test in adult case-finding in coeliac disease. *BMC Gastroenterol*. 2013;13:115.
- Arguelles-Grande C, Norman GL, Bhagat G, Green PHR. Hemolysis interferes with the detection of anti-tissue transglutaminase antibodies in celiac disease. *Clin Chem*. 2010;56:1034-1036.
- Wolf J, Hasenclever D, Petroff D, et al. Antibodies in the diagnosis of coeliac disease: a biopsy-controlled, international, multicentre study of 376 children with coeliac disease and 695 controls. *PLoS ONE*. 2014;9:e97853.
- Wolf J, Jahnke A, Fechner K, et al. Primate liver tissue as an alternative substrate for endomysium antibody immunofluorescence testing in diagnostics of paediatric coeliac disease. *Clin Chim Acta*. 2016;460:72-77.
- Farrell C-JL, Carter AC. Serum indices: managing assay interference. *Ann Clin Biochem*. 2016;53:527-538.
- Agarwal S, Vargas G, Nordstrom C, Tam E, Buffone GJ, Devaraj S. Effect of interference from hemolysis, icterus and lipemia on routine pediatric clinical chemistry assays. *Clin Chim Acta*. 2015;438:241-245.
- Lee KN, Birckbichler PJ, Fesus L. Purification of human erythrocyte transglutaminase by immunoaffinity chromatography. *Prep Biochem*. 1986;16:321-335.
- Wolf J, Petroff D, Richter T, et al. Validation of antibody-based strategies for diagnosis of pediatric celiac disease without biopsy. *Gastroenterology*. 2017;153:410-419.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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