OCCURRENCE OF SERUM ANTIBODIES AGAINST WHEAT ALPHA-AMYLASE INHIBITOR 0.19 IN CELIAC DISEASE

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Short title: **Antibodies to wheat alpha-amylase inhibitor 0.19 in celiac disease**

ABSTRACT

The alcohol-soluble fraction of wheat gluten (gliadins) induces in genetically susceptible individuals immunologically mediated celiac disease (CLD). However, gliadins and related cereal proteins are not unique foodstuff targets of CLD patients´ immune system. Non-gluten wheat alpha-amylase inhibitor 0.19 (AAI 0.19) has been found to be capable of activating human monocyte-derived dendritic cells and inducing pro-inflammatory status in intestinal mucosa of patients with celiac disease (CLD). The possible contribution of this reactivity in incomplete remission of CLD patients on a gluten-free diet (GFD) is matter of contention. In an attempt to characterize the antigenicity of AAI 0.19 in patients with active CLD, patients on a GFD and healthy controls we developed ELISA employing wheat recombinant AAI 0.19. Using this test we revealed a significant ($P<0.001$) elevation of IgA anti-AAI 0.19 antibodies (Ab) in patients with active CLD (12 out of 30 patients were seropositive) but also in CLD patients on a GFD (15/46), in contrast to healthy controls (2/59). Anti-AAI 0.19 IgG Ab levels were increased ($P<0.001$) only in patients with active CLD (14/30) in contrast to the controls. Interestingly, the levels of anti-AAI 0.19 IgG Ab were decreased in CLD patients on a GFD ($P<0.001$, 1/46) compared to the controls (1/59). Notably, 20 out of 30 of patients with active CLD were positive either for IgA or for IgG anti-AAI 0.19 Ab. Thus, the majority of CLD patients developed a robust IgA and IgG Ab response against AAI 0.19. These findings may contribute to the broadening of the knowledge about CLD pathogenesis.

Keywords

alpha-amylase inhibitor 0.19, celiac disease, gluten-free diet, IgA, IgG, ELISA

Abbreviations

CLD, celiac disease; GFD, gluten-free diet; C, healthy controls; AAI 0.19, alpha-amylase inhibitor 0.19; AAI 0.28, alpha-amylase inhibitor 0.28, IgA, immunoglobulin A; IgG, immunoglobulin G; IgE, immunoglobulin E; Ab, antibodies; AU, arbitrary units; O.D., optical density

1. INTRODUCTION

Cereals belong to the most important sources of nutrients in the world, with dominance of consumption in Europe and America. It should be noted, however, that wheat induces morbidity in over 2% of the word population. Generally, the main pathological conditions related with molecules of wheat grain are celiac disease (CLD), wheat allergy and non-celiac wheat sensitivity (Elli *et al.* 2015). The CLD affects nearly 1:100-200 of the wheat consumers in Europe, North America and North Africa; nevertheless, a substantial part of patients are undiagnosed due to clinically silent (asymptomatic) form(s) of the disease (Brar *et al.* 2006, Parada *et al.* 2011, Gujral *et al.* 2012, Kasarda 2013, Lebwohl *et al.* 2018). The CLD is induced in genetically susceptible individuals by ingestion of alcohol-soluble fraction of gluten (wheat-grain storage proteins) – gliadins and phylogenetically related cereals´ proteins: hordeins in barley, secalins in rye and certain avenins in oats. The alimentary intake of these proteins induces in CLD patients villous atrophy and crypt hyperplasia in duodenum and jejunum mucosa accompanied by malabsorption and gastrointestinal symptoms caused by the loss of digestive and barrier functions. The failure of oral tolerance to

cereal prolamins and elicitation of T-cell mediated autoimmunity are considered as pathological mechanisms of CLD. Active CLD is serologically characterized by production of antibodies (Ab) against induction agents of CLD – gliadins and various Ab against food antigens and autoantibodies, and a characteristic cytokine pattern. The testing of Ab against tissue transglutaminase (tTG) and deamidated gliadin or antibodies against endomysium is used in CLD diagnostics and verification of compliance to gluten-free diet (GFD), a sole rational life-long therapy of CLD. The adherence to GFD leads to healing of mucosal damage and disappearance of Ab against tTG, endomysium and gliadins (Catassi and Fasano 2010, Husby *et al.* 2012, Nevoral *et al.* 2014, Björck *et al.* 2015, Balakireva and Zamyatnin 2016, Wolf *et al.* 2017). Although a long-lasting and incomplete histological recovery, persistence of symptoms and discrepancy in serum levels of Ab against tTG and deamidated gliadin in CLD patients on a GFD may occur, the histological analysis of small-intestinal mucosa is not usually performed in a follow-up of these patients (Wahab *et al.* 2002, Tursi *et al.* 2003, Osman *et al.* 2014, Pekki *et al.* 2017, Burger et al. 2017). However, gliadins and related cereal proteins are not unique foodstuff targets of CLD patients´ immune system. The possible contribution of this reactivity in incomplete remission of CLD patients on a GFD is matter of contention. Huebener *et al.* (2015) described in CLD patients serum IgA and IgG Ab recognizing a number of non-gluten proteins extracted from U.S. hard red spring wheat *Triticum aestivum* Buttle 86 flour: serpins, purinins, globulins, farinins and several alpha-amylase/protease inhibitors. Interestingly, alpha-amylase inhibitor/trypsin inhibitor CM3 and alpha-amylase inhibitor (AAI) 0.19, a pest resistance molecule in wheat, were recently identified as potent activators of innate immune response in human monocyte-derived dendritic cells of both patients with active CLD and CLD patients on a GFD eliciting secretion of IL-8.

Consistently, enterobiopsy specimens from CLD patients in remission cultivated in a medium with alpha-amylase/trypsin inhibitors caused an increase of IL-8 mRNA expression. Moreover, these inhibitors stimulated also monocyte-derived dendritic cells of healthy controls to production of IL-12. The adjuvant effect of these molecules was mediated by their interaction with TLR4-MD2-CD14 complex (Junker *et al.* 2012). The AAI 0.19 and AAI 0.28 were originally described as allergens in baker´s asthma (Walsh and Howden 1989, Pfeil *et al.* 1990, Fränken *et al.* 1994, Amano *et al.* 1998). Subsequently, these AAIs were also identified as one of the major wheat allergens in wheat allergy (James *et al.* 1997, Zapatero *et al.* 2003, Šotkovský *et al.* 2008, Šotkovský *et al.* 2011, Kusaba–Nakayama *et al.* 2001).

The hydrosoluble allergens AAI 0.19 with adjuvant properties are structurally and physico-chemically different from water-insoluble gliadins. We hypothesized that AAI 0.19 could play a role in pathogenesis of CLD. Thus, we focused on analysis of the role of AAI 0.19 in CLD via the study of its antigenicity. In an attempt to characterize the antibody response to AAI 0.19 in patients with active CLD, patients on a GFD and healthy controls, we used an immunoblot technique employing the mixture of isolated wheat AAI 0.19 and 0.28, and developed a reproducible, robust ELISA test for quantification of serum IgA and IgG antibodies against AAI 0.19 protein.

2. MATERIAL AND METHODS

2. 1. Patients and healthy controls

The sera of 30 patients with active CLD (24 adults, 6 pediatric patients) were encompassed in our study. The group of adult patients comprised 16 women and 8 men with a mean age of 41.9 years, ranging from 21 – 76 years. Pediatric patients included four females and 2 males with mean age 6.8 years, range 3 – 13 years. The

CLD was diagnosed on the basis of modified ESPGAN criteria (Husby *et al.* 2012). The active CLD, i.e. CLD patients at the time of diagnosis, were positive for the serological CLD markers IgA anti-tissue transglutaminase (anti-tTG), IgA Ab and IgG anti-endomysial Ab (EMA) and Ab against deaminated gliadin. The pathological lesions in small bowel mucosa of these patients with active CLD were estimated at Marsh IIIA – IIIC. The Marsh IIIC grading was present in three, Marsh IIIB in 10 and Marsh IIIA in 11 out of 24 adult active CLD patients. Five out of six children patients met the new ESPGHAN guidelines (Husby et al. 2012) for omitting the small gut biopsy; these patients were symptomatic and highly seropositive for anti-tTG Ab (with titers of more than 10 times the upper limit of normal), positive for EMA and simultaneously possessing the HLA-DQ2.5 and/or DQ8 haplotypes. One child with CLD, positive for the CLD serological and genetic markers and manifesting gastrointestinal symptoms, was assessed as Marsh IIIA (male, 13 years).

The cohort of 46 CLD-GFD patients comprised 42 adults patients (31 women, 11 men) with mean age 39, ranging 19 – 77 years and four children (1 female, 3 male) with mean age 6.5 ranging 5 – 7 years with compliance to GFD for at least 12 months. All of these patients were seronegative for EMA and anti-tTG Ab, and free of CLD symptoms.

The control group consisted with 59 healthy individuals (28 women, 31 men), mean age 35.4, range 21 – 76 years. Individuals in the cohort were free of symptoms of gastrointestinal, autoimmune, inflammatory, malignant, allergic and infectious diseases and were seronegative for CLD markers.

The study was approved by the Local Ethics Committees from the Faculty Hospital Královské Vinohrady in Prague (Czech Republic) and the synlab czech Ltd.

149 (Czech Republic). Written informed consent was obtained from each participant in this
150 study.
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152 **2. 2. SDS-PAGE, Western blot analysis**
153 The protein separation was performed using sodium dodecyl sulfate
154 electrophoresis (SDS-PAGE) under reducing conditions as described by Laemmli and
155 Favre (1973). A mixture of isolated wheat AAI 0.19 and AAI 0.28, dominant components
156 of the "Alpha-amylase inhibitor from *Triticum aestivum* (wheat seed), Type III, A3535"
157 (Sigma-Aldrich, USA), was characterized by MALDI-TOF mass spectrometry on a
158 Ultraflex III instrument equipped with LIFT technology (Bruker Daltonics, Bremen,
159 Germany). The mixture of AAI 0.19 and AAI 0.28, and wheat recombinant AAI 0.19
160 (Apronex, Czech Republic) were initially dissolved in PBS at a concentration of 5 μg/μl
161 and 1 μg/μl, respectively (stock solution) and finally diluted 3:1 in sample buffer
162 containing 0.25 M Tris (Serva, Germany)(pH 6.8), 8% SDS (Serva), 40% glycerol
163 (Lachema, Czech Republic), 0.05 M Dithiothreitol (Sigma-Aldrich, USA) and 0.01%
164 bromophenol blue (Lachema). Samples of the mixture of AAIs in sample buffer were
165 boiled prior to separation due to better resolution and subsequently loaded into 15%
166 polyacrylamide gel in device Mini-Protean® 3 Cell device (Bio-Rad, USA) connected to
167 a EC 6000 – 90 power supply (EC Apparatus Corporation, USA) and separated under
168 electric conditions (35 mA, 150 V and 200 W) for 45 - 50 min. The sample of recombinant
169 wheat AAI 0.19 was not boiled prior to separation by SDS-PAGE under the same
170 conditions as the mixture of isolated wheat inhibitors. Separated proteins were
171 transferred to a nitrocellulose membrane (Amersham™Hybond™-ECL, GE Healthcare
172 Live Sciences, United Kingdom) in buffer containing glycine (192 mM, Serva), Tris (24.7
173 mM, Serva) and 20% methanol (Lach-Ner, Czech Republic) using the Trans-Blot (Bio-

Rad) and PowerPac™ Universal power supply (Bio-Rad) under 250 mA, 500 V and 200 W for 50 min. The nitrocellulose membrane was cut into strips. The strips were blocked with 2% non-fat powdered milk (ARTIFEX Instant, Czech Republic) in PBS containing 0.2% Tween 20 (Serva) (PBS-T (0.2%)) for 1-h at room temperature (RT) and then incubated with patients´ or control sera diluted at 1:500 (in case of IgA), 1:2500 (IgG) and 1:40 (IgE) in 1% non-fat powdered milk in PBS-T (0.2%) overnight at 4 °C. After washing with PBS-T (0.2%), the goat secondary peroxidase conjugated Ab against human IgA, IgG (The Binding Site, United Kingdom) diluted at 1:5000 or IgE (Invitrogen, USA) diluted at 1:10000 in 1% non-fat powdered milk in PBS-T (0.2%) was added. After 1-h incubation at RT and repeated washing, ECL reagent SuperSignal®West Pico (IgA, IgG) a SuperSignal®West Femto (IgE) (Thermo SCIENTIFIC, USA) and autoradiography (MXBE Film, Carestream Health France, France) were used for detection.

2. 3. Estimation of antibodies to alpha-amylase inhibitor 0.19

Wheat recombinant AAI 0.19 was used at a final concentration of 50 μg/ml in PBS. The 96-well polystyrene microtiter plates (Gama, České Budějovice, Czech Republic) were coated overnight at 4 °C. Blocking solution – 1% BSA (Sigma-Aldrich, USA) in phosphate buffered saline (PBS)(0.154 M NaCl, 1.4 mM $NaH_2PO_4.2H_20$, 3.35 mM $Na_2HPO_4.12H_2O$) was also used as a negative control. Patients´ and reference sera were diluted in blocking solution at 1:20 and 1:100 in case of detection of anti-AAI 0.19 IgA Ab and 1:100 and 1:500 in case of detection of anti-AAI 0.19 IgG Ab, and incubated overnight at 4 °C in wells of microtiter plates. Each dilution of patients´ and reference sera was tested in triplicate. The testing of anti-AAI Ab was performed in at least two independent experiments; the results obtained for individual serum were

averaged. After incubation, the plates were repeatedly washed with PBS and PBS containing 0.05% of Tween 20 (PBS-T (0.05%)) and subsequently peroxidase-labeled goat anti-human IgA or IgG Ab (The Binding Site) diluted at 1:750 in 10% normal goat serum (Sigma-Aldrich) in PBS (IgG) or in PBS containing 10% normal goat serum and 1% BSA (IgA) were added to the wells. After 1-h incubation at RT, the plates were repeatedly washed with PBS and PBS-T and the enzyme reaction was developed by adding a solution containing 3.87 mM o-phenylenediamine dihydrochloride (Sigma-Aldrich) in 0.1 M phosphate buffer (0.1 M $NaH_2PO_4.2H_20$, 0.1 M $Na_2HPO_4.12H_2O$, pH 6.0) containing 0.06% H_2O_2 (Chemapol, Czech Republic). The reaction was stopped by 2 M H_2SO_4 and optical density was read at 492 nm on a spectrophotometer Titertek Multiscan® MCC/340 ELISA Reader (Eflab, Finland) and BioTek® EL800 (BioTek, USA).

The internal laboratory standard (reference serum) was prepared from pooled celiac patients´ sera and was used in all ELISA tests. The serum levels of anti-AAI 0.19 Ab were expressed as arbitrary units (AU), represent the percentage of optical density (O. D.) of individual samples to O.D. of reference serum. Cut-off value, a threshold above which we take the individual positive, was calculated as the mean + double standard deviation of levels of IgA or IgG anti-AAI 0.19 in healthy donor group (healthy controls).

2. 4. Statistical analysis

The Ab levels are usually not directly proportional to antigen-binding capacity of serum samples (Arranz and Ferguson 1993). In most cases, non-parametric tests are appropriate for analysis of immunohaematological data (Reverberi 2008). We analyzed for Gaussian distribution of anti-AAI 0.19 Ab levels in all cohorts by D´Agostino &

Pearson omnibus normality test and Shapiro-Wilk normality test. Using the tests a non-Gaussian distribution of IgA or IgG anti-AAI 0.19 Ab levels was revealed in groups of patients with CLD and CLD-GFD patients. For this reason, we used Mann-Whitney U test for comparison of Ab levels between groups.

3. RESULTS

3. 1. Western blot analysis of antigenicity of AAI 0.19 and AAI 0.28 using a mixture of isolated wheat proteins

In the first stage of our study of occurrence of anti-AAI Ab in CLD we estimated, by Western blotting with a mixture of isolated wheat AAI 0.19 and AAI 0.28 separated by SDS-PAGE, serum IgA, IgG and IgE Ab reactivity in active CLD patients, CLD-GFD patients and healthy controls (Figure 1, Table 1). Employing this technique, we detected reactivity of IgA anti-AAI 0.19 and/or anti-AAI 0.28 in eight out of 30 active CLD patients, in five out of 46 CLD-GFD and five out of 59 healthy controls. Moreover, also 13 out of 30 active CLD, three out of 46 CLD-GFD patients and 5 out of 59 healthy individuals were seropositive for IgG isotype of these Ab. Surprisingly, the IgE Ab recognizing AAI 0.19 and/or AAI 0.28 were also found in 12 out of 30 CLD, in six out of 46 CLD-GFD, and 5 out of 59 healthy controls.

3. 2. ELISA for quantification of IgA and IgG Ab against wheat recombinant AAI 0.19

The purpose of ELISA was to precisely characterize the antigenicity and compare the serum levels of IgA and IgG Ab (isotypes associated with active CLD) against AAI 0.19 in patients with active CLD, CLD on a GFD and healthy controls. The capability of binding of serum IgA (Figure 2A) and IgG Ab (Figure 2B) to recombinant

wheat AAI 0.19 was verified by titration analyses. The slope of the titration curve of Ab of the majority of tested sera was similar, indicating their similar specificity for AAI 0.19. For better resolution of the ELISA test, we used two dilutions of tested sera for quantification of the level of anti-AAI 0.19 Ab. We estimated the optimal dilution of sera for testing Ab against wheat recombinant AAI 0.19 in the ELISA at 1:20 and 1:100 for IgA and 1:100 and 1:500 for IgG Ab. The results from individual dilutions of sera samples (in triplicate) were averaged.

Comparison the seropositivity and serum levels of IgA and IgG Ab against AAI 0.19 in patients with active CLD, CLD on a GFD and healthy controls is given in Table 2 and Figure 3. The ELISA detected statistically significantly elevated ($P<0.001$) IgA anti-AAI 0.19 Ab in patients with active CLD (117.2 ± 105.3 AU, mean ± standard deviation) and even in CLD-GFD (80.1 ± 43.6 AU) in contrast to healthy controls (50.5 ± 24.3 AU). Although we detected reduced average level of IgA anti-AAI 0.19 Ab in a cohort of CLD-GFD in comparison with active CLD, the difference between the values of averages of the Ab levels was not statistically significant. The IgA serum levels of anti-AAI 0.19 in 12 out of 30 CLD patients, in 15 out of 46 CLD-GFD patients and in two out of 59 healthy controls exceeded cut-off value (99 AU), above which we take the individual seropositive. The IgG Ab were significantly ($P<0.001$, 149 ± 78.4 AU) elevated only in patients with active CLD, while they were significantly decreased in CLD-GFD patients ($P<0.001$, 59 ± 37.1 AU) when compared to both patients with active CLD and healthy controls (82.7 ± 33.7 AU). The 14 out of 30 CLD patients, one out of 46 CLD-GFD patients and one out of 59 healthy controls were seropositive for IgG anti-AAI 0.19 Ab (cut-off value 150 AU). Taken together, six out of 30 active CLD patients were seropositive for both isotypes of anti-AAI 0.19 Ab. However, 20 out of 30 CLD patients were seropositive either for IgA or for IgG anti-AAI Ab. The IgA and IgG

Ab reactivity of CLD patients, those on a GFD and healthy controls (C) with recombinant wheat AAI 0.19 was confirmed using Western blot (Figure 4).

4. DISCUSSION

In genetically susceptible individuals, nutrient components may induce an immunologically-mediated food intolerance or hypersensitivity. Wheat amylase/trypsin inhibitors belong to the ubiquitous group of small naturally occurring pest-resistance proteins (Ryan 1990, Cordain 1999). Recently, wheat AAI 0.19 was identified as a potent activator of human monocyte-derived dendritic cells of both patients with active CLD and CLD patients on a GFD, and in healthy controls via interaction with the TLR4-MD2-CD14 complex (Junker *et al.* 2012). Subsequently, the antigenicity of alpha-amylase/protease inhibitors for CLD patients was detected in the study of Huebener *et al.* (2015). The TLR4-mediated adjuvant effect of amylase/trypsin inhibitors in gluten-containing samples (irrespectively whether baked or otherwise processed) induced infiltration and activation of myeloid cells and release of inflammatory mediators in intestinal mucosa of experimental mice (Zevallos *et al.* 2017). Moreover, wheat amylase/trypsin inhibitors were suggested as causative agents of non-celiac wheat sensitivity through activation of patients´ immune system via the TLR4 (Schuppan and Zevallos 2015). These inhibitors have been known for years to induce a rapid increase of proinflammatory cytokines and chemokines in CLD patients in remission after a duodenal and rectal wheat challenge (Kontakou *et al.* 1995, Chowers *et al.* 1997). However, no information is available on the adaptive immune response against these amylase/trypsin inhibitors in CLD patients.

In the present study, we focused on characterizing Ab against wheat AAI 0.19 (and AAI 0.28) in CLD patients and those on a GFD for the first time. Using Western

blot with a mixture of isolated wheat AAIs as antigens, we found relatively high frequency of CLD patients seropositive for anti-AAI 0.19 and/or anti-AAI 0.28 IgA, IgG, and IgE Ab. Consequently, we confirmed these results for IgA and IgG isotypes using quantitative ELISA. For this purpose, we developed ELISA employing recombinant wheat AAI 0.19 as an antigen. Despite the fact that AAI 0.19 represents a negligible part of wheat grain, 20 out of 30 CLD patients were seropositive either for IgA or for IgG isotype of anti-AAI Ab. The frequency and distribution of individual values of IgA and IgG Ab against AAI 0.19 in our study suggest a genetically controlled predisposition to immune response against AAI 0.19, which is reinforced by natural adjuvant effect described by Schuppan and Zevallos (2015). On the other hand, we can also assume the contribution of impaired barrier function of CLD patients´ intestine to the development of anti-AAIs Ab enabling increased penetration of the AAIs (and other food antigens) through mucosa.

High levels of IgA and IgG Ab against AAI 0.19 in patients with CLD document advanced immune response of long duration indicating a germinal center reaction in lymphoid follicles and cooperation with antigen-specific CD4+ T cells. In general, it is assumed that the high antigen-Ab (B-cell receptor) avidity complexes promote extrafollicular B-cell response and increase plasma cell generation (Chan and Brink 2012). Consistent with this, the extrafollicular response or a short-time germinal reaction is assumed in the development of tTG specific B-cells (and the production of low avidity autoAb). It could be triggered by self-oligomerization of this CLD autoantigen (Gelderman *et al.* 2014, Stamnaes *et al.* 2015). Remarkably, the capability of oligomerization is also characteristic for alpha-amylase inhibitors; the AAI 0.19 naturally occur as (homo)dimer in solution (Buonocore *et al.* 1984, ODA *et al.* 1997).

Although long-lasting (more than 1 year) adherence to GFD in CLD patients led to disappearance of Ab against gliadins and tTG (serological markers of the CLD) indicating compliance to the diet in all patients, some of them remain positive for IgA Ab against non-gluten AAI 0.19 protein in our study. The persistence of these Ab may be explained by the presence of a low amount of AAI 0.19 in the diet and its potent immunostimulatory effect on mucosal immune system of patients with CLD, represented predominantly by IgA isotype. The small hydrophilic molecule of AAI 0.19 could be present in deproteinized grain starch utilized for GFD diet (Täufel *et al.* 1996, Gazza *et al.* 2016). On the other hand, the phenomenon of residual and selective Ab reactivity against AAI 0.19 in CLD patients on a GFD is hardly explicable as a difference between the dynamics of IgA and IgG anti-AAI 0.19 isotypes. However, it could be partly explained as part of homeostatic mechanisms including isotype switching induced by increased expression of IL-10 and TGF-β as a consequence of homeostatic mechanisms during the exclusion of residual, harmless, antigen at mucosal surfaces.

Eventually, the immune reactivity against the AAI 0.19 (and other AAIs and amylase/trypsin inhibitors) could also be the results of cross-reactivity induced by molecular mimicry between the AAI 0.19 and other foodstuff constituents or components of altered intestinal microbiota, which is considered to play important role in CLD pathogenesis (Verdu *et al.* 2015). The AAI 0.19 was originally described as allergen in baker´s asthma and wheat allergy (Walsh and Howden 1989, Pfeil *et al.* 1990, Fränken *et al.* 1994, Amano *et al.* 1998, James *et al.* 1997, Zapatero *et al.* 2003, Šotkovský *et al.* 2008, Šotkovský *et al.* 2011, Kusaba–Nakayama *et al.* 2001). Hence, production of IgG and IgA Ab against AAI 0.19 could be a physiological response preventing allergic reaction in some CLD patients. None of the CLD patients in our

study has allergy symptoms and all patients possess a physiological level of serum IgE. For this reason we can hypothesize that the initial stage (or active) of CLD, associated with damage of small gut mucosa, involves also the production of specific IgE due to elevated levels of IL-4 (Manavalan et al. 2010). The pathogenic mechanism of allergic reaction and its typical dynamics is probably suppressed or mitigated by the presence of IgA and IgG anti-AAI 0.19 Ab. Finally, the IgA anti-AAI 0.19 Ab perseverance in CLD patients on GFD could be partially caused by Ab cross-reactivity of mucosal B-cells with a structurally similar antigen/autoantigen. The role of anti-AAI 0.19 Ab is not known but the effect of various isotypes of anti-AAI Ab can be different. The IgA and IgG Ab could interfere with the stimulation of antigen presenting cells by AAI 0.19 and can block the epitopes for IgE Ab and in such away prevent allergy reaction. Though the AAI 0.19 and AAI 0.28 have been known as allergens for many years, key IgE epitope sequence has been proposed only for 0.28 AAI (amino acids 9 – 26). The epitope structure of AAI 0.19 is not satisfactorily characterized. Interestingly, regardless of 60% sequential similarity between these two inhibitors, the amino acid homology between N-terminal parts of these proteins, which in AAI 0.28 is immunodominant for IgE Ab of patients suffering from wheat and related allergy, is approximately only 33% (Walsh and Howden 1989). Our results, however, clearly indicate a strong antigenicity of AAI 0.19 for CLD patients and some of the healthy individuals. Interestingly, recently used selection criteria in breeding programs for new, high-yield wheat varieties prefer an increased amylase/trypsin inhibitors content in wheat grain due to improving plant pest-resistance (Ryan 1990, Cordain 1999, Sands *et al.* 2009, Boukid *et al.* 2017).

In conclusion, our work contributes to characterization of antigenicity of wheat non-gluten protein AAI 0.19, which possesses adjuvant properties for CLD patients

and is the allergen in wheat allergy and baker´s asthma. In any event, the production of IgA and IgG against this protein in some CLD patients suggests advanced and clinically significant immune reaction against this food component. The relatively high prevalence of Ab against wheat non-gluten allergen AAI 0.19 justifies future analysis of the role of these Ab and AAI 0.19 in CLD and in general population. What remains for the analysis of anti-AAI 0.19 Ab role and diagnostic value is to characterize also the occurrence of Ab against AAI 0.19 in diseases associated with CLD and allergic diseases.

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543

7. TABLE

7. 1. Table 1

Fraction of seropositive individuals for antibodies against wheat alpha-amylase inhibitor 0.19 and/or 0.28 detected by Western blot

Cohorts	IgA	IgG	IgE
CLD	8/30 (27%)	13/30 (43%)	12/30 (40%)
CLD-GFD	5/46 (11%)	3/46 (~ 7%)	6/46 (13%)
Healthy controls	5/59 (~ 8%)	5/59 (~ 8%)	5/59 (~ 8%)

CLD, celiac disease; CLD-GFD, CLD on a gluten-free diet; IgA, IgG, IgE: antibody isotypes; number of seropositive individuals/total number in cohort

7. 2. Table 2

Seropositive individuals for antibodies against recombinant wheat alpha-amylase inhibitor 0.19 detected by ELISA

Cohorts	IgA	IgG
CLD	12/30 (40%)	14/30 (~ 47%)
CLD-GFD	15/46 (~ 33%)	1/46 (~ 2%)
Healthy controls	2/59 (~ 3%)	1/59 (~ 2%)

CLD, celiac disease; CLD-GFD, CLD on a gluten-free diet; IgA, IgG: antibody isotypes; number of seropositive individuals (Ab levels exceeding the cut-off value)/total number in the cohort

8. LEGENDS TO FIGURES (FIGURE CAPTIONS)

8. 1. Figure 1

Examples of reactivity of IgA, IgG and IgE serum antibodies (Ab) of celiac (CLD) patients, those on gluten-free diet (CLD-GFD) and healthy controls (C) with isolated wheat alpha-amylase inhibitor 0.19 and alpha-amylase inhibitor 0.28. Proteins were separated by 15% polyacrylamide gel with sodium dodecyl sulfate electrophoresis

(SDS-PAGE), stained with Coomassie brilliant blue R-250 and subsequently blotted into nitrocellulose membrane. ST, molecular weight standards (kDa); lane 1, SDS PAGE of alpha-amylase inhibitors 0.19 (~ 15 kDa) and truncated 0.28 (~ 11 kDa); lane 2, Ponceau S stained Western blot of separated inhibitors transferred into the membrane; lanes 3-5: IgA Ab reactivity of CLD patients; lanes 6,7: IgA Ab reactivity of CLD-GFD; lanes 8,9: weak reactivity of IgA Ab of C; lanes 10-12: IgG Ab reactivity of CLD patients; lanes 13,14: IgG Ab reactivity of CLD-GFD; lanes 15,16: reactivity of IgG Ab of C; 17-19: IgE Ab reactivity of CLD patients; lanes 20,21: reactivity of IgE Ab of CLD-GFD; lanes 22,23: weak reaction of IgE Ab of C. Negative controls (without employing patients or control serum) represent only anti-IgA Ab peroxidase labeled Ab (lane 24), anti-IgG peroxidase labeled Ab (lane 25) and anti-IgE peroxidase labeled Ab (lane 26).

8. 2. Figure 2

Titration curves of serum IgA (**A**) and IgG (**B**) antibodies (Ab) against recombinant wheat alpha-amylase inhibitor 0.19 in active celiac patients (CLD 1-6), healthy controls (C 1-4) and celiac patients on a gluten-free diet (CLD-GFD). O.D.: optical density, dilution of sera is indicated at horizontal axis.

8. 3. Figure 3

Distribution of individual serum levels of IgA and IgG antibodies (Ab) against recombinant wheat alpha-amylase inhibitor 0.19 (AAI 0.19) in patients with celiac disease (CLD), CLD on a gluten-free diet (CLD-GFD) and healthy controls (C). Horizontal lines indicate the mean serum levels of specific antibodies in cohorts. AU,

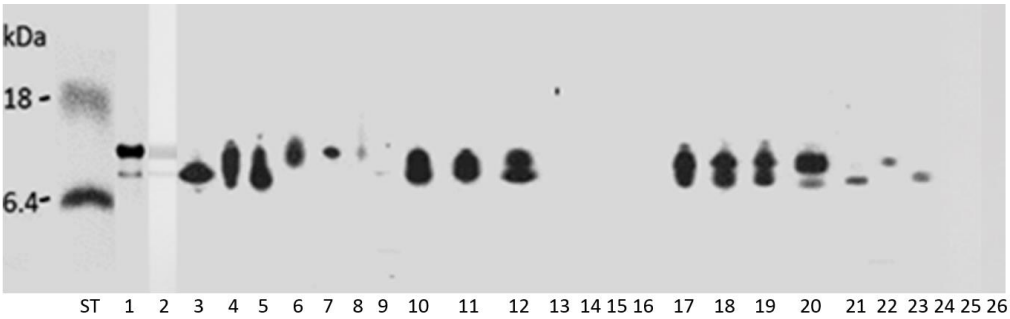
arbitrary units; n, number of patients; ***, P<0.001; NS, not significant. Cut-off value for IgA anti-AAI 0.19 Ab is 99 AU and for IgG anti-AAI 0.19 Ab 150 AU.

8. 4. Figure 4

Verification of IgA and IgG antibodies (Ab) reactivity of celiac patients (CLD), CLD patients on a gluten-free diet (CLD-GFD) and healthy controls (C) with recombinant wheat alfa-amylase inhibitor 0.19 using Western blot. ST, molecular weight standards (kDa); Lane 1 indicates position of recombinant wheat alfa-amylase inhibitor 0.19 in SDS-PAGE electrophoretogram, stained by Coomassie brilliant blue R-250. The image of inhibitor blotted into nitrocellulose membrane and visualized by Ponceau S is localized in lane 2. The intensity of IgA Ab reactivity of CLD patients with wheat alpha-amylase inhibitor 0.19 is demonstrated in lanes 3-5 (CLD), lanes 6, 7 (CLD-GFD), and lanes 8, 9 (C). Lane 10 indicates negative control – immunoblot with only peroxidase-conjugated anti-human IgA Ab. Examples of IgG Ab reactivity with the inhibitor are indicated in lanes 11-13 (CLD) and lanes 14, 15 (CLD-GFD). Lanes 16 and 17 document non-reactive IgG Ab of healthy controls. Lane 18 represents negative control – immunoblot with only peroxidase-conjugated anti-human IgG Ab.

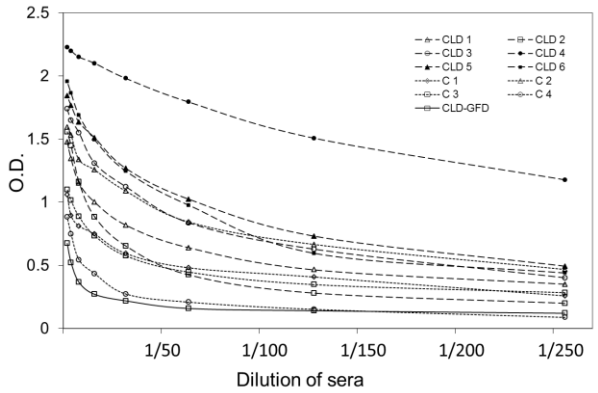
9. FIGURE GRAPHICS

9. 1. Figure 1



9. 2. Figure 2

A



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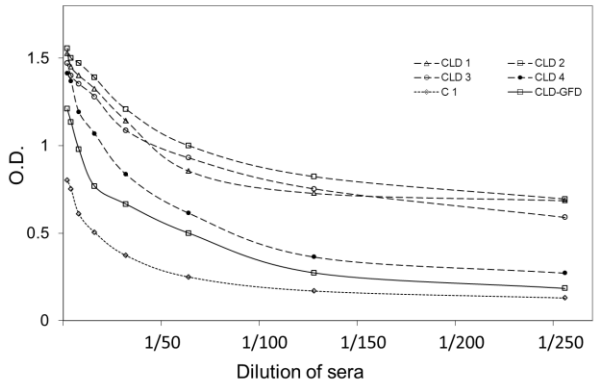
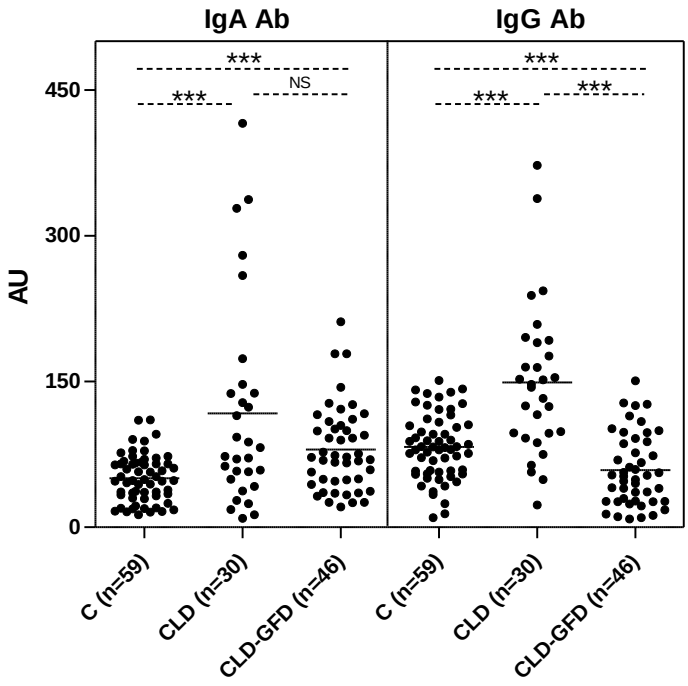


9. 3. Figure 3



9. 4. Figure 4