INTRODUCTION

Celiac disease (CeD) is caused by an autoimmune reaction to gluten in genetically susceptible individuals. While classical CeD is characterized by intestinal symptoms as diarrhea, malabsorption and anorexia, patients with atypical CeD have less intestinal symptoms but may suffer from extra-intestinal symptoms such as fatigue and anemia (1). Although patients with CeD have increased levels of IgA and IgG to gliadins, it is the IgA (or IgG in IgA-deficient patients) autoantibody to Ca²⁺-activated tissue transglutaminase 2 (TG2), whether detected in serum and/or as intestinal IgA–TG2 immune complexes in the lamina propria, that defines CeD (2). Celiac disease is treated by a lifelong gluten-free diet, which results in the disappearance of intestinal lesions, TG2 autoantibody, and antibodies to deamidated gliadin. Increased anti-gliadin
IgG serum levels are not CeD specific and are often observed in non-celiac individuals, they are useless in CeD diagnostics (3).

Celiac disease-specific developmental enamel defects (CeD-DEDs) are symmetrical and chronological defects that predominantly affect permanent teeth (4). The number of teeth affected, the topical location and the characteristics of the defects are generally considered to reflect CeD activity during amelogenesis as a gluten-free diet restores normal amelogenesis (5). The severity of DEDs caused by CeD ranges (according to the Aine classification) from hypomineralized, discolored spots (Grade 1) to severe substance defects (Grade 4) (6). The etiology of CeD-DEDs is unclear and is presumably multifactorial as malnutrition (7), genetics (8,9), CeD-associated immune activity and autoimmunity may be contributory factors (10–12).

Autoimmune reactions may be induced by molecular mimicry, in which structural similarity between foreign antigens and host proteins allows cross-reactive B cells to receive T-cell help and become disease drivers (13). Gliadin and amelogenin share amino acid sequences, which may partly explain the cross-reactivity between anti-amelogenin IgG and gluten (11). Although it remains to be clarified whether anti-amelogenin IgG is involved in the etiology of CeD-DEDs, the functional implication of the cross-reactivity would depend on the amelogenin regions to which the IgG reacts. We previously reported the epitope mapping of anti-AMELX IgA in children with untreated CeD (12). The current paper is an extended epitope-mapping of serum anti-amelogenin IgG in children with newly diagnosed CeD and disease controls selected from the same two cohorts.

MATERIAL AND METHODS

Study population

Patient cohorts: Some of the patients (n = 23) studied here were included in a previous study in which the IgA anti-AMELX epitopes were mapped (12). The present cohort 1 included blood samples collected for diagnostic purposes were obtained from 73 children (median age 8 years; age range 1–16 years) with biopsy-proven, but untreated, CeD and from 25 children (median age 8 years; age range, 1–17 years) proven by biopsy not to have CeD. An additional 31 serum samples had been collected at different time points from nine children in cohort 1 proven by biopsy not to have CeD (median age at sampling was 5 years; age range 2–12 years; all from the Paediatric Department, Drammen Hospital, Norway). All samples in cohort 1 were from children on a gluten-containing diet. Diagnosis was based on the level of anti-TG2 IgA, HLA-DQ genotype, clinical examination, and histopathological and immunohistochemical examination of jejunal biopsy samples. Blood sampling was conducted as described in detail previously (12). Cohort 2 comprised 73 children with untreated CeD: all had sera containing anti-TG2 IgA titers >10 times the upper normal limit that had been sent to Oslo University Hospital, Ullevaal, Norway, to be examined anti-endomysium IgA reactivity. The study was approved by the Regional Committees for Medical and Health Research Ethics (REK, Norway). However, as the sera in cohort 2 were anonymized, we were unable to obtain any clinical information on the participants other than that they all had a serum level of anti-TG2 IgA that was >10 times the upper normal limit (confirmed on two occasions) and demonstrated anti-endomysium IgA reactivity, and therefore were diagnosed with CeD without confirmation by intestinal biopsy, according to the ESPGHAN 2012 diagnostic criteria (www.epsghan.org).

Sera from all patients (146 with CeD and 34 non-CeD controls) were tested for IgG reactivity to amelogenin, using porcine amelogenin (Emdogain; Straumann) as antigen in ELISA, as detailed elsewhere (11). The following samples (n = 42) from patients with CeD were selected for further epitope mapping, specifically: samples from patients in cohorts 1 (n = 14) and 2 (n = 22) with anti-amelogenin IgG immune reactivity higher than the median value; samples from patients with enamel defects (cohort 1, n = 3); and samples from three patients who had participated in the anti-AMELX IgA study (12) (these patients were included despite having anti-amelogenin IgG levels lower than the median). The control consisted of the five sera with the highest levels anti-amelogenin IgG and the five blood samples with the highest levels of anti-amelogenin IgG, from 10 controls (cohort 1).

Peptide synthesis

Potential linear B-cell epitopes in AMELX isoform-1 (Q99217-1; UniProt database) were predicted using the online BepiPred epitope prediction program (http://www.cbs.dtu.dk/services/BepiPred/). The four most probable epitope areas were chosen based on protein hydrophobicity.

Three peptide sets were used in epitope mapping. Two peptide sets were used to map the anti-AMELX IgG epitopes (Figure 1) (12). In short, the first peptide set consisted of 19 overlapping biotinylated 12-13mer peptides with four amino acids offset (Sigma-Aldrich) covering the central AMELX_{75-150} region. The following peptides were also included in the first peptide set: peptides A_{2-12} and B_{28-41}, corresponding to the N-terminal region of AMELX-1; peptide W_{161-175} corresponding to the hydrophilic C terminus; peptide AMELX-2_{(AAFAMPVLTLKP}_{2}, corresponding to the exon-3-deleted AMELX isoform-2 (Q99217-2); and peptide AMELX-3_{[FSYENSHSQAINVRATAVLTP]} corresponding to the exon-4 (in bold)-expressing AMELX isoform-3 (Q99217-3).
A second peptide set covered the rest of the AMELX isoform-1 sequence, namely AMELX peptides AB13-27, AC23-32, AD39-54, AE150-163, AA (AAFA MPLPPHPGHPG YNFSEYVL TPLKWYQSIR PPYSYGYPE MGGWHLHHQI PVLSQHQHPPT HTLQPHHHHP) (containing the signaling peptide cutting site), and AF (SIRPPPLPPPML), which is specific for the leucine-rich amelogenin peptide (LRAP). Peptides AA-4-7, AB12-27, and AC23-32 were used to examine whether the anti-AMELX IgG epitopes were localized in AMELX isoforms 1, 2, or 3. This peptide set also clarified if reactivity to peptide B28-41 was directed to AMELX isoform-1 or to LRAP, by analyzing reactivity to peptides B28-41, AC23-32, AD39-54, AE150-163, and AF28-32:150-155, as detailed elsewhere (12).

The third peptide set, containing eight peptides, was used to fine map the reactivity to peptide AB and AMELX-3. The YEVLTPL sequence in peptide-AB13-27 is similar to a hemagglutinin amino acid sequence in both the common (KDVLTP) and even more so in a rare (Y DVLTPL) morbilivirus variant. (The shared pentapeptide, VLTP is in bold.)
italic indicate that the amino acid glutamic acid (E) in peptide-AB is a conservative substitution for the aspartic acid (D) in the morbillivirus hemagglutinins, whereas the underlined K denotes that the amino acid lysine (K) is a radical substitution compared to the amino acid tyrosine (Y). The morbillivirus used in measles vaccines expresses, most presumably, the common K<sup>D</sup>VLTPL-containing hemagglutinin variant, which is a known morbillivirus hemagglutinin glycoprotein B-cell epitope, according to the Immune Epitope Database and Analysis Resource (IEDB) (https://www.iedb.org/). The AMELX-3 peptide containing the exon 4 sequence, NSHSQAINVDRTAL, was also included in the third peptide set in addition to two peptides overlapping either the exon-3/exon-4 (FSYENSHIS) or the exon-4/exon-5 (RTALVLTP) boundary (exon-4 sequences are shown in bold). The sequence, Q<sub>64</sub>PHHHIPV<sub>71</sub>, was additionally included to ensure that the whole AMELX isoform-1 sequence was covered.

**Epitope mapping and data interpretation**

Peptide mapping was performed as previously described, with minor revisions (5). The wells of Streptavidin Coated
High Capacity ELISA plates (Thermo Fisher Scientific) were coated with 1 μg per well of biotinylated peptides diluted in Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 and 0.1% BSA and incubated overnight (4°C), then washed in TBS buffer containing 0.05% Tween 20 and 1.25% BSA (TBS/Tween/1.25%BSA). Duplicate samples of blood (diluted 1/50, 1/80, or 1/100 in TBS/Tween/1.25%BSA) and serum (diluted 1/200 or 1/400) were added to wells coated with biotinylated peptides, incubated for 2 h at room temperature (RT = 20-22°C), then washed before addition of horse-radish peroxidase-conjugated goat anti-human IgG (1/8000; 30 min, Southern Biotech). The reaction was visualized following addition of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma), stopped by addition of H₂SO₄, and the results were analyzed at 450 nm on an Epoch microplate spectrophotometer (BioTek). Peptides from the first and second sets were analyzed at 450 nm on an Epoch microplate spectrophotometer (BioTek). Peptides from the first and second sets were analyzed at 450 nm on an Epoch microplate spectrophotometer (BioTek). Peptides from the first and second sets were tested simultaneously, and data were normalized by subtracting the mean absorbance of the six weakest peptide reactions (lowest quartile used as background) to obtain corrected data that were then divided with the background absorbance value to obtain fold increase above background (14); a two-fold increase was used as the cut off for positivity. Analysis of the results obtained from serially diluted blood samples revealed that the normalization also adjusted for dilution-induced increases in both background and signal, which made it possible to compare data obtained for different blood/sera concentrations (results not shown).

**Antigen-inhibition experiments**

One-hundred and forty milligrams of cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare) was washed in 10 ml of 1 mM HCl, then centrifuged (5 min, 1000 g, 20°C) and the supernatant discarded. This washing procedure was repeated five times. The washed Sepharose was then mixed with 700 μl of 3.6 mg ml⁻¹ gliadin (G3375; Sigma-Aldrich) in 0.1 M carbonate buffer (pH 10) containing 0.5 M NaCl and 25% dimethylformamide, to increase gliadin solubility.

Emdogain (Straumann) was diluted with 0.05 M acetic acid, incubated at 4°C overnight, and centrifuged (10 min, 16,000 g, 4°C). Four-hundred and ninety microliters of this solution (2.2 mg ml⁻¹ of Emdogain) was added to 140 μg of washed Sepharose and mixed, then 210 μl of concentrated coupling buffer (0.33 M carbonate, 1.67 M NaCl, pH 10) was added. The initial Emdogain solution has a low pH to keep the amelogenin content monomeric until addition of carbonate buffer to induce coupling.

The Sepharose and antigen mixtures were incubated for 2 h at room temperature with rotation (Mini Rotating Hybridization Incubator, HIM20; Grant Instruments) at ~10 rpm, then washed and blocked following the manufacturer's recommendations.

Thirty microliters of Sepharose (alone, or coated with gliadin or Emdogain) per milliliter of diluted serum or blood, as used in the ELISA epitope mapping, was incubated overnight at 4°C in a Mini Rotating Hybridization Incubator (HIM20) at ~10 rpm and then centrifuged (5 min, 850 g, 20°C). The supernatants were collected and used for solid-phase antigen absorption-induced reduction in IgG reactivity to the peptides AB₁₃–₂₇, B₂₈–₄₁, E₇₅–₈₆, L₁₀₃–₁₁₄, O₁₁₅–₁₂₆, R₁₂₇–₁₃₈, U₁₃₉–₁₄₀, AE₁₅₀–₁₆₃, and AMELX-3.

Residual reactivity was defined as the difference in absorbance between the sample preincubated with gliadin- or Emdogain-coated Sepharose, and the sample absorbed with uncoupled (but blocked) Sepharose.

**In situ detection of amelogenin in rat incisor**

A rat mandibular arcade was fixed overnight at 4°C in periodate-lysine-based fixative containing 1.25% paraformaldehyde and then decalcified in 10% EDTA/100 mM Tris, pH 7.0, at 4°C with agitation (25 rpm; Gyro-Rocker), changing the decalcifying solution twice a week. X-ray examination was used to monitor the decalcification process, which was complete after 12 d. The decalcified mandible was rinsed in PBS, twice, each time for 45 min at 4°C with agitation, then 20% sucrose was added and allowed to infiltrate the mandible for 1 h at 4°C. After separating the mandible, a perpendicular cut was made between molar one (M1) and molar two (M2). The part containing the secretory ameloblasts was further trimmed, keeping M2 and molar three (M3) and all of the posterior part of the incisor. The specimen was embedded in Optimal Cutting Temperature medium (OCT; CellPath) before being snap-frozen in liquid nitrogen and stored at −70°C. Three serially cut cryosections (4 μm) were placed on each microscope slide, dried overnight at room temperature, wrapped in aluminum foil, and stored at −20°C. The sections were thawed, left overnight at 40°C, acetone fixed for 10 min at room temperature, then incubated in a periodate-lysine-based fixative supplemented with 1.25% paraformaldehyde for 10 min at 4°C, and rinsed in destilled water prior to immunohistofluorescence staining. The sections were then incubated, overnight at 4°C, with sera (diluted 1/200 and 1/500 in TBS/Tween/1.25% BSA) from five highly anti-Emdogain/amelogenin IgG-reactive patients with CeD, one anti-Emdogain/amelogenin IgG-negative patient with CeD, and one anti-Emdogain/amelogenin IgG-negative control patient without CeD. This primary incubation was followed by incubation for 1 h at room temperature with rabbit anti-human IgG (diluted 1:1000 in TBS/Tween/1.25% BSA; Dako), then by incubation for 30 min with Alexa 594-conjugated goat anti-rabbit IgG (diluted 1:5000 in TBS/Tween/1.25% BSA; Invitrogen). Serial cut sections were incubated with rabbit
polyclonal anti-human amelogenin IgG (diluted 1:100 in TBS/Tween/1.25% BSA, Santa Cruz Biotechnology) and processed as above. Three of the serum samples from patients with CeD and the two control samples (diluted 1:250 in TBS/Tween/1.25% BSA) were additionally mixed with a monoclonal antibody to amelogenin (diluted 1:100 TBS/Tween/1.25% BSA, clone 6G3; IgG2b, Sigma-Aldrich) and processed using a three-color immunofluorescence technique, where Alexa-488-conjugated goat anti-mouse IgG2b (diluted 1:1000 in TBS/Tween/1.25% BSA; Invitrogen) and 4′,6-diamidino-2-phenylindole (300 nM; Molecular Probes) were added to the Alexa 594-conjugated goat anti-rabbit IgG (see above). The sections were then incubated for 30 min with this antibody combination to identify patient IgG binding to positively identified amelogenin-containing structures and to visualize the cell nuclei. The immunohistofluorescence-stained slides were analyzed in a Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with a plan-neofluar 40×/1.3 oil lens and appropriate fluorochrome filters.

RESULTS

All samples selected for epitope mapping from children with CeD (42/42) and almost all controls (9/10) reacted to at least one peptide. Samples from more than half of the children with CeD (cohorts 1 and 2) demonstrated increased IgG reactivity to the AMELX peptides AB13-27, B28-41, and AE150-163, and ≥25% (cohort 2 only) demonstrated strong reactivity to peptide U139-150 (the AMELX-3 peptide) and to peptides E75-86, L103-114, O115-126, and V143-155 (Figure 2A,B). Almost all (9/10) children with no CeD (controls) showed reactivity to the N-terminal peptide AB13-27, nine reacted to C-terminal peptide AE150-163, and six reacted to N-terminal peptide B28-41. The other peptides were detected only sporadically (Figure 2C). The immune response of each patient to each peptide is collectively visualized in Figures 3 and 4.

Although both IgA (12) and IgG recognized many of the same peptides (Table 1), IgA reacted more strongly to the central AMELX peptide I, and IgG reacted more strongly to peptides B, AE and, to a certain degree, AMELX-3, than the corresponding IgG.

Reactivity to central peptides and the C-terminal peptide, AE150-163

Thirty-four (81%) of 42 samples from children with CeD demonstrated ability to detect peptide AE150-163, of which 17 (50%) also reacted to the upstream peptide U139-150. Thirteen (38%) demonstrated ability to recognize the 6mer overlapping peptide V141-155, and six (18%) demonstrated ability to recognize the LRAP-specific upstream peptide AF28-32:150-155 (Figure 5A). Although samples from seven of the children with CeD reacted to the non-overlapping peptide T135-146, which shares the PLPPM154 sequence with peptides AE150-163, U139-150, V141-155, and AF28-32:150-155, only two samples reacted to all these peptides, suggesting that flanking regions are important and/or that additional epitopes exist within each peptide. However, 13 patients with CeD had IgG immune reactivity to peptide AE150-163 but no reactivity to the other peptides, suggesting that this peptide contains at least two epitopes including the exclusive PLPPM154 sequence in peptide AE150-163 (Figure 5A).

Fine mapping the IgG reactivity to peptide AB13-27

Nine of the 10 controls without CeD and 39 (93%) of the 42 children with CeD showed reactivity to peptide AB13-27. However, only three of these peptide-AB-reactive children with CeD, and none of the controls (without CeD), reacted to the downstream, overlapping peptide AC23-32 with the common sequence 25LKWYQ27 (Figure 5B).

Fourteen (36%) of the children with CeD and two (22%) of the nine controls, all of whom reacted to peptide AB13-27, also reacted to AMELX-3 (which shares both the VLTP and FSYE sequences with peptide AB13-27), but only one of the children with CeD reacted to the VLTP-containing AMELX-2. Thus, the peptide AB epitope may depend on either or both of the shared VLTP and FSYE sequences. If so, then the 14mer-long exon-4 (NSHSQAINVDRTAL) that is inserted between the FSYE and VLTP sequence in peptide AMELX-3, may allow its tertiary structure to mimic the linear structure of peptide AB. Alternatively, reactivity to the epitope may have been dependent on differences in the VLTP N-flanking sequences. (Figure 5B). Interestingly, peptide AB13-27 contains a sequence of seven amino acids, 13YEVLTPL23, that is partly included in the known morbillivirus hemagglutinin B-cell epitope, SIEHQVKDVLTP15,16, in which the biochemical properties of aspartic acid (D) are similar to those of the glutamic acid (E) that flanks the VLTP sequence in peptide AB. Another morbillivirus strain has a hemagglutinin sequence – Y DVLTPL – that is even more similar to peptide AB. Yet another similar sequence (PDVLTPL) is also found in the non-gluten wheat starch granule proteins. We therefore attempted to perform a more precise epitope-mapping by using sera from six children with CeD and two controls (no CeD) that had a sufficiently high IgG response to peptide AB13-27 to warrant further epitope mapping. However, none of the peptide AB13-27-reactive sera demonstrated IgG reactivity to any of these short peptides (data not shown).
**FIGURE 3** Sera from the children in cohort 1 (n = 17), with apparently less severe celiac disease (CeD) than those in cohort 2, reacted predominantly to peptides containing sequences found in the N or C terminus of amelogenin, X isoform (AMELX) (AB13-27, B28-41, and AE150-163), as well as the AMELX isoform-3-specific peptide, AMELX-3. Each child had an individual pattern of reactivity. Only peptides for which reactivity was more than twice the background are displayed. Peptides are presented on the x-axis, the patients (each shown in a different color) are presented along the y-axis and normalized absorbance values for each peptide are shown on the z-axis.

**FIGURE 4** Sera from the children in cohort 2 with anti-transglutaminase 2 (TG2) IgA levels more than 10 times higher than the upper normal limit (n = 25), and apparently more severe celiac disease (CeD) than those in cohort 1, detected peptides containing sequences found in the N terminus and central region of amelogenin, X isoform (AMELX), as well as the AMELX isoform-3-specific peptide, AMELX-3. Each child had an individual pattern of reactivity. Only peptides for which IgG reactivity was more than twice the background are displayed. Peptides are presented on the x-axis, the patients (each shown in a different color) are presented on the y-axis, and normalized absorbance values for each peptide are shown on the z-axis.
IgG anti-AMELX-peptide reactivity after gliadin and amelogenin preabsorption

Samples of sera showing high reactivity to AMELX peptides were antigen-absorbed by preincubation with gliadin- and Emdogain-coated Sepharose beads, and then retested on high-reactivity peptides. Gliadin preabsorption reduced anti-peptide E75-86 reactivity by >98%, to a median value of 1.15% (range 0%–17%), anti-peptide L103-114 reactivity to a median value of 10% (range 5%–25%), anti-peptide O115-126 reactivity to a median value of 4% (range 0%–14%), anti-peptide R127-138 reactivity to a median value of 7% (range 0%–21%), and anti-peptide U139-150 reactivity to a median value of 17% (range 0%–37%) (all median values are relative to the original reactivity of each peptide). Similarly, pretreatment with Emdogain (porcine amelogenin) reduced anti-peptide E75-86 reactivity to a median value of 1.25% (range 0%–65%), anti-peptide L103-114 reactivity to a median value of 20% (range 0%–56%), anti-peptide O115-126 reactivity to a median value of 3% (range 0%–32%), anti-peptide R127-138 reactivity to a median value of 7% (range 0%–50%), and anti-peptide U139-150 reactivity to a median value of 26% (range 0%–63%) (all median values are relative to the original reactivity of each peptide) (Figure 6B).

Immunohistochemistry

All sera from children with CeD, with high anti-Emdogain IgG reactivity, reacted to amelogenin in the rat incisor (Figure 7A,D), as confirmed by the identical staining pattern with rabbit (not shown) or murine monoclonal antibody to amelogenin (Figure 7B,E). Sera from a child with CeD, but with no anti-Emdogain IgG reactivity (Figure 7G), and from a control child (with no CeD) (not shown) did not produce any specific immune reaction.

DISCUSSION

Petronijevic et al reported that children with untreated CeD and approximately 20% of children with no CeD have increased IgA and IgG titers to amelogenin (11). Moreover, although anti-Emdogain/amelogenin IgA titers were found to be more CeD specific than the corresponding IgG titers, the anti-Emdogain/amelogenin IgG immune response
was significantly stronger in children with the most severe CeD who had intestinal Marsh 3b–c lesions (11). The anti-gliadin IgG titers correlated to the anti-Emdogain/amelogenin IgG titers, suggesting the samples contained anti-gliadin/amelogenin cross-reactive antibodies (11), which the current study confirmed was to the central amelogenin region. However, some of the anti-Emdogain/amelogenin IgG reactivity appeared not to be a result of anti-gliadin cross-reactivity (11), which the epitope mapping in the current study revealed to be the N- and/or C-terminal regions. Moreover, serum IgG from Emdogain-high titer CeD children, but not from low-titer CeD children or controls, showed ability to detect amelogenin in situ in the pre-enameal, similarly to what SÓÑORA et al, reported for adult women with CeD (17). To what extent anti-AMELX IgG may interfere with normal amelogenesis would depend on the amelogenin epitope(s) to which these antibodies bind. Both gliadin cross-reactive and gliadin-independent anti-amelogenin IgGs may interfere with normal enamel production if they access the enamel organ and interact with critical amelogenin regions.

The IgG response to the peptides L, O, R, T, U, and V from the central region of AMELX was much stronger in children with CeD than in non-CeD controls (12) and appeared to be associated with disease activity as the proportion of children with CeD reacted to all of the peptides containing the sequence PLPPM (shown in bold) but not to the peptides R and S, which contain the sequence PLPP (shown in bold), pointing to the M in PLPPM as critical for this epitope. (B) Sera from 39 of 42 children with CeD and from nine of 10 control children without CD showed reactivity to peptide AB. Only one serum sample (from a child with CeD) reacted to the amelogenin, X isoform (AMELX) isoform-2-specific peptide, AMELX-2, which contains the common VLTP sequence, and only three samples reacted to peptide AC, which contains the common KKYQP sequence. However, 14 (36%) of the 39 serum samples from children with CeD and two (22%) of the nine serum samples from controls that showed reactivity to peptide AB reacted to the AMELX isoform-3-specific peptide, AMELX-3, that shares both VLTP and FSYE sequences with peptide AB. Thus, reactivity to AMELX-3 may depend on a conformational epitope that mimics the linearized FSYE-VLTP sequences in peptide AB.
Normal matrix metalloproteinase-20 (MMP-20)-degraded amelogenin may have an important additional function as the central amelogenin P103\textsubscript{45-147} fragment binds to, and protects, MMP-20-activated transforming growth factor-beta1 (TGF-β1) from further degradation, which allows TGF-β1 to remain active during the secretory phase as the TGF-β1–amelogenin–P103 complex activates the TGF-β receptor 1 and is only slowly cleaved by kallikrein-4 during the maturation stage (21). IgG to the central AMELX region may therefore either inhibit TGF-β1 binding, leaving it open for further degradation by MMP-20, or bind the AMELX–TGF-β1 complex and prevent kallikrein-4-induced degradation. The latter would increase TGF-β1 activity in the post secretory stages, which induced pitted and hypoplastic enamel in a murine TGF-β1 transgene model (22).

The C-terminal peptide AE\textsubscript{150-163} is adjacent to the C-terminal telopeptide that is important for nanosphere self-assembly (21). Surprisingly, preabsorption on gliadin- or Emdogain-coated Sepharose did not reduce anti-peptide AE\textsubscript{150-163} IgG reactivity. Whereas gliadin has little sequence similarity to peptide AE\textsubscript{150-163}, the sequence of AE (PLPPMLPDLTLEAW) is similar to, but with some important differences (shown in bold) from the porcine amelogenin sequence (PLPPMFSMQSLPDLPLEAW). In addition to a single amino acid difference (T\textsubscript{159}P, bold and underlined in the sequences shown in the previous sentence), the porcine amelogenin sequence contains the FSMQSL sequence at the region that would correspond to human AMELX M\textsubscript{154}–L\textsubscript{155}. Although Emdogain predominantly contains the 20-kDa p148mer porcine amelogenin isoform lacking the C terminus, the linear hydrophobic C-terminal AE epitope could nevertheless have been hidden within the tertiary folded Emdogain, both of which may explain why preincubation with antigen-coated Sepharose, suggesting that these epitopes are not exposed in native folded, Sepharose-bound full-length proteins. Closed circles are blood samples from cohort 1, open squares are serum samples from cohort 2. GLD, sera preincubated with gliadin-coated Sepharose; EMD, sera preincubated with Emdogain-coated sepharose.

**FIGURE 6** Gliadin cross-reactivity. (A) Preincubation of blood/sera from patients with celiac disease (CeD) with gliadin- or Emdogain-coated Sepharose beads significantly reduced IgG reactivity to the amelogenin, X isoform (AMELX) peptides containing sequences to the central area of AMELX (E, L, O, R, and U) compared with control (uncoated) Sepharose, suggesting the presence of gliadin cross-reactive antibodies. (B) Reactivity to peptides AB, B, and AE, and to the AMELX isoform-3-specific peptide (AMELX-3) was not reduced after preincubation with antigen-coated Sepharose, suggesting that these epitopes are not exposed in native folded, Sepharose-bound full-length proteins. Closed circles are blood samples from cohort 1, open squares are serum samples from cohort 2. GLD, sera preincubated with gliadin-coated Sepharose; EMD, sera preincubated with Emdogain-coated sepharose.
In the present study, most of the children with CeD and controls had IgG to the N-terminal peptides AB13-27 and B28-41. However, the level of IgG was independent of disease activity (Marsh grading, data not shown) and not reduced by preincubation with gliadin- or Emdogain-coated Sepharose, presumably because these regions become hidden in the globular pockets during amelogenin polymerization, as previously discussed (12). BLAST analysis of peptide AB13-27 revealed that its YEVTLP sequence was similar to a sequence in the morbillivirus virus hemagglutinin B-cell epitope, ID 58485 (https://www.iedb.org/epitope/58485). The high frequency of anti-peptide AB13-27 IgG reactivity both in children with and without CeD could have been caused by morbillivirus vaccine-induced anti-hemagglutinin reactivity. However, further epitope mapping failed to identify the true nature of these reactions as serum IgG did not react to the short 7mer morbillivirus hemagglutinin sequences, to the 7mer morbillivirus homology sequence in peptide AB13-27, or to the similar sequence in starch granule proteins. Reactivity of IgG to linearized sequences in the N-terminal peptides AB and B may nevertheless result in the binding of unfolded AMELX in vivo and prevent proper amelogenin folding. The N terminus is also involved in amelogenin interactions.

**Figure 7** Reactivity of IgG in sera from children with celiac disease (CeD) to amelogenin in sections of rat incisor. Sera from two children with CeD (A, D, arrows, red) reacted to the amelogenin-containing enamel as identified by murine monoclonal antibody (mAb) to amelogenin (B, E, arrows, green). (G) Serum from a child with CeD, but ELISA negative for anti-Emdogain IgG reactivity, showed no reactivity to amelogenin (arrow). Whereas sera from children with CeD produced a staining pattern identical to that of the mAb to amelogenin (C, F, yellow), the superimposed image from the control children (who did not have CeD) revealed no IgG reactivity to amelogenin (red) in the amelogenin-stained, green fluorescence (I, arrow). The primitive enamel is seen between the layer of ameloblasts (Am) and the layer of dentin (De). Multicolor microphotograph (×250) showing staining for anti-amelogenin IgG autoantibodies (red) in two children with CeD with high serum titers of anti- amelogenin IgG (A, D) and for amelogenin (green) (B, E, H) and the results obtained for one control child (without CeD) (G, I). The nuclei (blue) are visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI).
to itself, to other enamel matrix proteins, ameloblasts (27), and nanoribbons formation (28), and peptide AB Y17/E18 is an MMP-20 cutting site (29). Thus, antibody binding to linear sequences in the N terminus may impair several functions of amelogenin.

Fourteen children with CeD and one control had IgG reactivity to the exon-4-containing peptide, AMELX-3. One child with CeD, with Aine grade 2 enamel defects, had particularly high IgG reactivity to peptide AMELX-3. Unfortunately, we could not clinically examine the other 13 AMELX-3-reactive children with CeD, partly because of ethical restrictions for children in cohort 2 (we were only allowed to examine the sera from children in cohort 1 (n = 6), who presumably had a more severe CeD, in an anonymous manner) and partly because of insufficient contact information. This combination of IgG reactivity to peptide AMELX-3 and enamel defects was also observed in one other patient with CeD, who had enamel defects and particularly high levels of serum IgA (but not of IgG) to peptide AMELX-3 (12). Exon-4-expressing amelogenin (AMEL4+) is only present in the early maturation stage (30), and despite not being abundantly present in human developing enamel (31), it may regulate enamel development as mutation leading to AMEL4+ overexpression induced amelogenesis imperfecta (AI) (32).

Although antibodies to amelogenin may interfere with normal amelogenin function, it is unclear how the antibodies may enter the site of enamel crystallization during amelogenesis. Both the enamel organ itself and the ameloblasts are supposedly sealed by tight junctions (33). However, tight junctions may loosen up during the late secretory stage as approximately 25% of the ameloblasts autolyze prior to the maturation stage (33), allowing serum immunoglobulin to enter the enamel organ, which may explain why Pierce et al (34) observed IgE on post secretory ameloblasts during tooth eruption. Moreover, gluten-induced intestinal inflammation results in increased intestinal leakiness through interferon-γ-induced production of epithelial zonulin that disassembles tight junctions in untreated CeD. Furthermore, the 20mer α-gliadin peptides (QVLQQSTYQLLQELCCQHLW and QQQQQQQQQQIQLQQILQQ) activate the innate immune system through interaction with the epithelial chemokine receptor CXCR3/MyD88, leading to further zonulin release and increased gut permeability (35). Similar effects may occur in distant sites as patients with untreated CeD have increased levels of circulating cytokines, gluten peptides (36), and zonulin (35). Thus, in children with severely CeD, both gluten-induced increase in circulating cytokines, zonulin, and gluten peptides may loosen tight junctions in distant places as in the enamel organ and facilitate IgG entrance.

The lack of clinical data connecting the epitope-mapping results with enamel defects in the majority of the patients is a limitation of our study. The enamel defects may also depend on CeD severity and age at diagnosis in an anti-amelogenin epitope-dependent manner. Moreover, antibodies are often directed to conformational epitopes, which limits the epitope repertoire examined in the current study. Longer peptides may mimic some of the tertiary epitopes that are lost in shorter peptides, as illustrated in the loss of peptide AB13-27 reactivity using shorter peptides. In the present study, we detected some of the amelogenin epitopes that may be involved in disturbing normal amelogenesis in CeD, and only further studies may reveal the true etiology of CeD-specific enamel defects.

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AUTHOR CONTRIBUTIONS

SP and TSH designed the study, contributed to the running of study, recruited patients, analysed results, and reviewed and contributed to the writing of manuscript; SS contributed to the study design, performed and reviewed the analysis, and reviewed and contributed to the writing of the manuscript.

CONFLICT OF INTERESTS

The authors have declared no conflicts of interest.

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