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To cite this article: Anne Lammi, Pekka Arikoski, Arja Hakulinen, Ursula Schwab, Matti Uusitupa, Seppo Heinonen, Erkki Savilahti, Tuure Kinnunen & Jorma Ilonen (2016) Development of gliadin-specific immune responses in children with HLA-associated genetic risk for celiac disease, *Scandinavian Journal of Gastroenterology*, 51:2, 168-177, DOI: [10.3109/00365521.2015.1067328](https://doi.org/10.3109/00365521.2015.1067328)

To link to this article: <http://dx.doi.org/10.3109/00365521.2015.1067328>



Published online: 10 Jul 2015.



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## ORIGINAL ARTICLE

**Development of gliadin-specific immune responses in children with HLA-associated genetic risk for celiac disease**ANNE LAMMI<sup>1</sup>, PEKKA ARIKOSKI<sup>2</sup>, ARJA HAKULINEN<sup>3</sup>, URSULA SCHWAB<sup>4,5</sup>,  
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**Abstract**

**Objective.** The development of gliadin-specific antibody and T-cell responses were longitudinally monitored in young children with genetic risk for celiac disease (CD). **Material and methods.** 291 newborn children positive for HLA-DQB1\*02 and -DQA1\*05 alleles were followed until 3–4 years of age by screening for tissue transglutaminase autoantibodies (tTGA) by using a commercial ELISA-based kit and antibodies to deamidated gliadin peptide (anti-DGP) by an immunofluorometric assay. Eighty-five of the children were also followed for peripheral blood gliadin-specific CD4<sup>+</sup> T-cell responses by using a carboxyfluorescein diacetate succinimidyl ester-based *in vitro* proliferation assay. **Results.** The cumulative incidence of tTGA seropositivity during the follow-up was 6.5%. CD was diagnosed in nine of the tTGA-positive children (3.1%) by duodenal biopsy at a median 3.5 years of age. All of the children with confirmed CD were both IgA and IgG anti-DGP positive at the time of tTGA seroconversion and in over half of the cases IgG anti-DGP positivity even preceded tTGA seroconversion. Peripheral blood T-cell responses to deamidated and native gliadin were detected in 40.5% and 22.2% of the children at the age of 9 months and these frequencies decreased during the follow-up to the levels of 22.2% and 8.9%, respectively. **Conclusions.** Anti-DGP antibodies may precede tTGA seroconversion and thus frequent monitoring of both tTGA and anti-DGP antibodies may allow earlier detection of CD in genetically susceptible children. Peripheral blood gliadin-specific T-cell responses are relatively common in HLA-DQ2-positive children and are not directly associated with the development of CD.

**Key Words:** antibodies to deamidated gliadin peptide, celiac disease, children, gliadin-specific T cells, tissue transglutaminase antibodies

**Introduction**

Celiac disease (CD) is a chronic T-cell-mediated autoimmune enteropathy induced by dietary gluten in genetically susceptible individuals. Over 90% of CD patients express the HLA-DQ2 molecule encoded by the HLA-DQA1\*05 and -DQB1\*02

alleles [1,2]. Most of the remaining patients express either the HLA-DQ8 molecule encoded by the DQA1\*03 and DQB1\*0302 alleles, or have at least one of the two alleles forming the risk-associated DQ2 molecule, DQB1\*02 or DQA1\*05 allele [3]. The risk for CD is further increased by carrying two copies of the HLA-DQ2-encoding alleles,

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(Received 27 April 2015; revised 23 June 2015; accepted 24 June 2015)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2015 Informa Healthcare  
DOI: 10.3109/00365521.2015.1067328

emphasizing the role of HLA-DQ2 as the most important immunogenetic factor [4,5]. The prevalence of CD is approximately 1% in Western Europe and North America but screening studies suggest significantly higher prevalence rates than what is observed when the diagnosis is made by clinical suspicion [6–8]. Currently, the screening of CD is based on IgA class tissue transglutaminase autoantibody (tTGA) measurement and the diagnosis is confirmed by the typical histological changes, crypt hyperplasia and villous atrophy observed in the duodenal biopsy of small intestine [9]. Recently proposed new criteria by The European Society for Paediatric Gastroenterology, Hepatology and Nutrition, however, suggest that the diagnosis of CD in symptomatic children can be based on high levels of CD-specific antibodies reducing the need for duodenal biopsy [10].

Although the screening of CD has been for some time based on the measurement of IgA class tTGA, it has been demonstrated that especially IgG-class antibodies to deamidated gliadin peptide (anti-DGP) may appear earlier than tTGA-seropositivity in genetically susceptible children [11,12]. This is in accordance with the suggested significance for T-cell responses to DGPs in the intestinal mucosa in the initiation of the disease process [13]. Detection of T-cell responses to deamidated gliadin in the peripheral blood is an interesting concept for monitoring CD-related immunity, since their emergence might even precede the antibody response to gliadin peptides, and thus they could be used as the first detectable marker of the disease process. In several studies, T cells specific to deamidated gliadin have been demonstrated in the circulation of adult patients with CD as a marker of active disease process, most commonly after oral gluten challenge [14–16]. Recently, we were able to demonstrate the presence of T cells specific to deamidated gliadin in the peripheral blood of children with active CD without gluten challenge [17]. Circulating T cells specific to various DGPs were also detected in a high percentage of children with newly diagnosed CD by Liu et al. [18].

The aim of this study was to monitor the development of gliadin-specific antibody and T-cell responses in children genetically at-risk for CD and to evaluate whether these responses can be detected before the clinical onset of the disease. For this, we prospectively followed newborn children carrying the high risk-associated combination of HLA-DQB1\*02 and -DQA1\*05 alleles (HLA-DQ2) until 3–4 years of age by screening for tTGA and anti-DGP antibodies as well as by assessing gliadin-specific CD4<sup>+</sup> T-cell responses in the peripheral blood.

## Materials and methods

### Study design

The current follow-up study was performed in a cohort of Finnish children with a high genetic risk for CD (recruited at the Kuopio University Hospital and the Kätilöopisto Maternity Hospital in Helsinki). Parents of newborn children were first met at the Maternity Hospital and the study protocol was briefly described to them. If the family decided to participate in the study the cord blood sample of the newborn was analyzed for the presence of HLA-DQ2, as described earlier [19,20]. The result of the HLA-screening was informed to the families by a phone call when the child was 1–2 weeks old. Written informed consent was obtained from all families. Families also participated in a study where special nutritional counseling was given to half of the families with the purpose to optimize the introduction of gluten into a time-window between 4 and 6 months age together with ongoing breast feeding. No significant effect of the counseling for these parameters was detected. The study plan was accepted by the ethics committee of the Kuopio University Hospital.

A total of 2013 children were screened for risk-associated HLA-DQ2. Altogether 339 of the children (16.8%) were positive for HLA-DQ2 and finally 291 children continued in the follow-up study (Figure 1). Blood samples were collected from the children at the ages of 9, 12, 18, 24 and 36 months, and also at 48 months for the children who reached that age during the follow-up. Sera were analyzed for tTGA and anti-DGP antibodies. Eighty-five children (from the Kuopio University Hospital cohort) were additionally followed for circulating gliadin-specific T-cell responses until 48 months of age. Results of the tTGA tests were reported to the participating families and the parents of tTGA-seropositive children were offered a possibility to undergo duodenal biopsy to confirm the diagnosis of CD. In 7 of the 10 cases where duodenal biopsy was performed the child had a high tTGA level (>10 times the upper limit of normal, median 100, range 8.2–100) [10]. The final diagnosis was based on typical histological findings, villous atrophy and crypt hyperplasia, in the duodenal biopsy of small intestine detected in all children with a diagnosis of CD [9].

### Antibody assays

All collected serum samples were kept frozen at –80°C until analyses. Anti-DGP antibodies were measured by using the time-resolved immunofluorometric assay (TR-IFMA), which allows the simultaneous detection

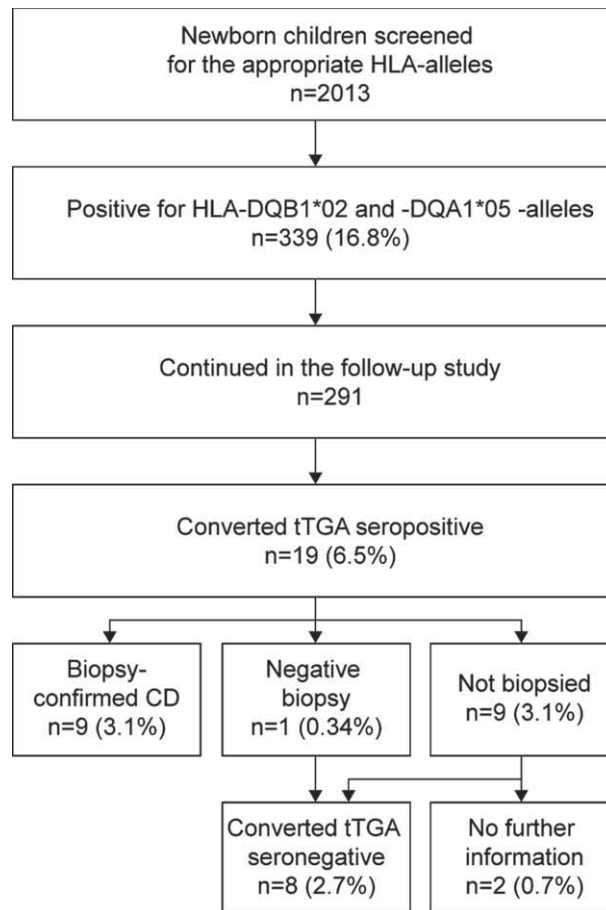


Figure 1. Study design. Children positive for HLA-DQB1\*02 and -DQA1\*05 alleles were prospectively followed for the emergence of tissue transglutaminase autoantibody (tTGA) seropositivity and development of celiac disease until the age of 48 months.

of IgA and IgG class antibodies to a synthetic peptide, derived from gamma gliadin of wheat [21]. The cut-off value used for positivity was 153 for IgA and 119 for IgG class anti-DGP antibodies, as determined earlier [12]. A commercial ELISA-based kit was used for measuring tTGA (EliA Celikey, Phadia, Freiburg, Germany). Values >8 U/ml were considered positive, as suggested by the manufacturer. Only IgA class tTGA was measured.

#### *T-cell proliferation assay using the CFSE dilution method*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Molecular Probes, Carlsbad, CA, USA) in order to detect cell proliferation, as described in detail earlier [17]. Labeled PBMCs were suspended in culture medium at  $10^6$ /ml and stimulated with various antigens in a volume of 200  $\mu$ l in 96-well round-bottomed plates (Costar, Corning Incorporate, Corning, NY, USA).

Deamidated gliadin (gTG; 20  $\mu$ g/ml) was prepared by incubation with tissue transglutaminase (tTG), as described earlier [17]. Native gliadin alone was used at a final concentration of 10  $\mu$ g/ml and tTG at 2  $\mu$ g/ml. Purified tetanus toxoid (TT, National Institute of Health and Welfare, Helsinki, Finland) was used as an independent control antigen at a final concentration of 1  $\mu$ g/ml and purified phytohaemagglutinin (PHA, Remel, UK) as an independent mitogen control of cell functionality at 2  $\mu$ g/ml. Cell cultures were maintained at 37°C in a 5% CO<sub>2</sub> incubator in 6–8 equal wells, PHA in 2–4 equal wells. After 10 days of culture, replicates for each antigen were pooled, washed with ice-cold wash buffer (phosphate buffered saline, 2% fetal calf serum and 0.1% NaN<sub>3</sub>) and stained with the following antibodies: anti-CD4-PerCP-Cy5.5, anti-CD45RO-PE-Cy7, anti-CD45RA-APC and integrin  $\beta$ 7-PE (BD Pharmingen, San Jose, CA, USA). Staining for cell surface markers was carried out on ice in the dark for 20 min, followed by two washes. The samples were analyzed on a FACS Canto II flow cytometer (BD, Mountain View, CA, USA). CD4<sup>+</sup> T-cell proliferation was

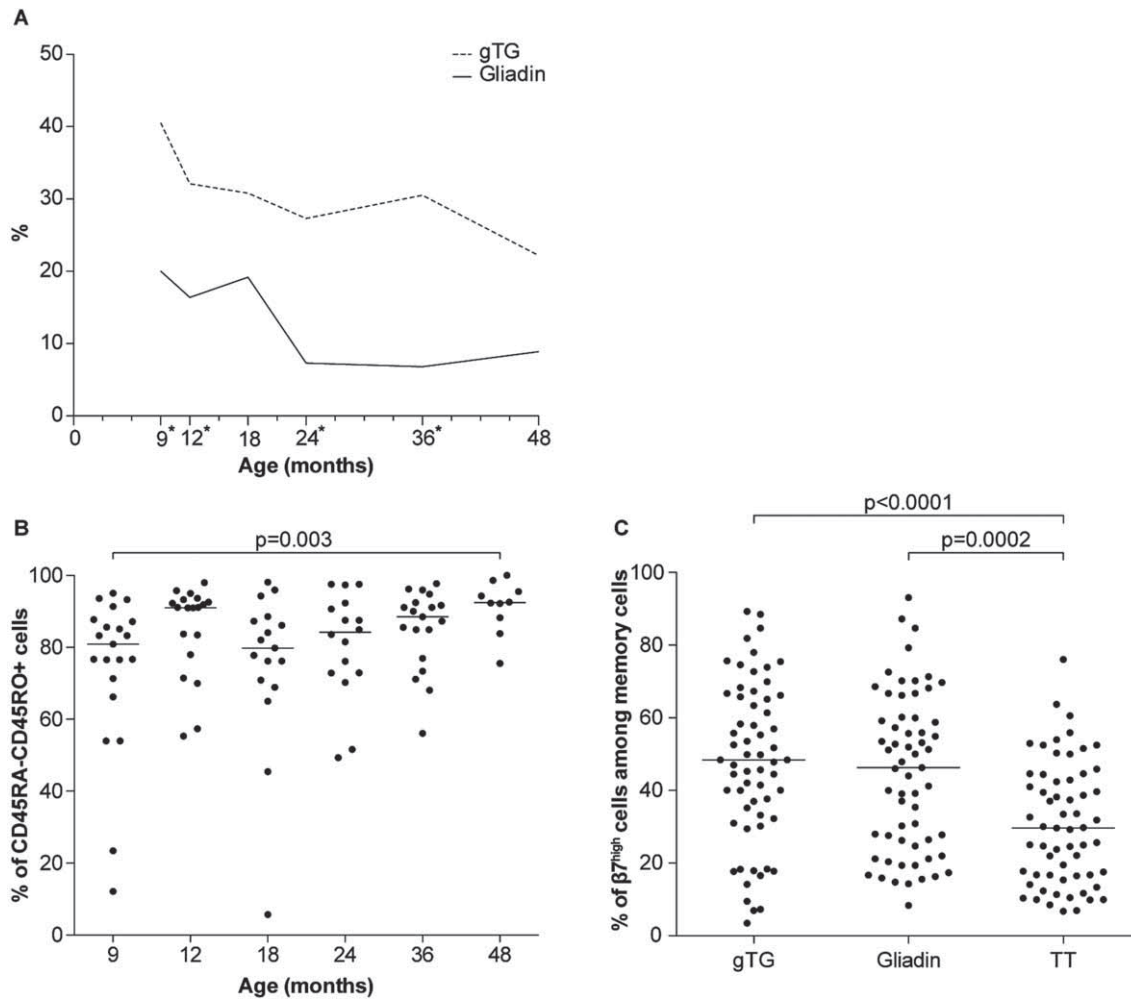


Figure 2. (A) The frequency of positive peripheral blood CD4<sup>+</sup> T-cell responses to gTG and native gliadin decreased during the follow-up. (B) The percentage of memory phenotype (CD45RA-CD45RO<sup>+</sup>) cells within the CD4<sup>+</sup> CFSE<sup>low</sup> population in cultures stimulated with gTG. (C) The percentage of  $\beta 7$ -integrin expressing cells within proliferating memory CD4<sup>+</sup> T cells stimulated with gTG, gliadin and TT. Deamidated gliadin (gTG), native gliadin (Gliadin), tetanus toxoid (TT). \* $p < 0.05$ . All the statistical analyses were conducted by using the Mann-Whitney U-test.

measured by determining the percentage of CFSE<sup>low</sup> cells within the CD4<sup>+</sup> T-cell fraction. The cell division index (CDI) was calculated as follows: percentage of CD4<sup>+</sup>CFSE<sup>low</sup> cells in cultures stimulated with antigen/CD4<sup>+</sup>CFSE<sup>low</sup> cells in cultures without

antigen [22]. Individual responses to an antigen were considered positive when the CDI was  $\geq 2.0$  and the difference in the percentage of CD4<sup>+</sup>CFSE<sup>low</sup> cells between stimulated and unstimulated cultures was at least 0.5%.

Table I. The frequency of peripheral blood T-cell responses to deamidated gliadin (gTG), gliadin, tissue transglutaminase (tTG) and tetanus toxoid (TT) at different time points in all tested children ( $n = 85$ ), as assessed by the CFSE dilution method.

Age (months)	Frequency of positive T-cell responses (%)			
	gTG	Gliadin	tTG	TT
9	17/42 (40.5%)*	8/40 (20.0%)	2/30 (6.7%)	26/32 (81.3%)
12	18/56 (32.1%)*	9/55 (16.4%)	0/39 (0%)	29/39 (74.4%)
18	16/52 (30.8%)*	10/52 (19.2%)	1/40 (2.5%)	23/41 (56.1%)
24	15/55 (27.3%)*	4/55 (7.3%)	0/42 (0%)	28/43 (65.1%)
36	18/59 (30.5%)*	4/59 (6.8%)	0/48 (0%)	41/48 (85.4%)
48	10/45 (22.2%)	4/45 (8.9%)	3/38 (7.9%)	23/38 (60.5%)

\* $p < 0.05$  compared with native gliadin, Fisher's exact test.

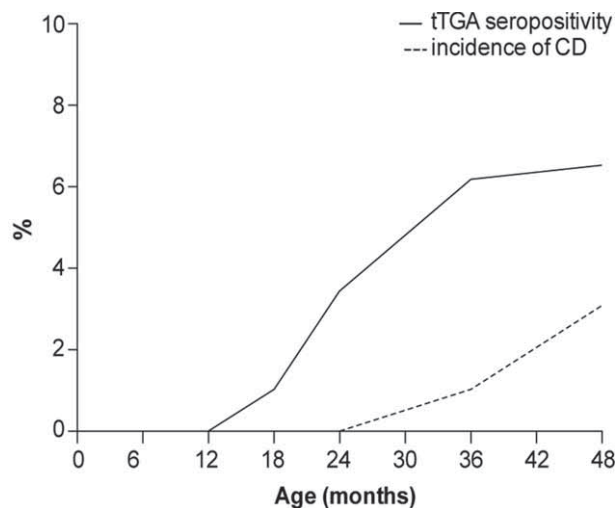


Figure 3. The cumulative percentage of tTGA seropositivity and the incidence of celiac disease during the follow-up.

### Statistical analysis

All statistical analyses were performed by GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The Mann–Whitney U-test was used to compare the median levels of tTGA at the time of seroconversion between children not biopsied and/or turned seronegative and children with biopsy-proven CD. The Mann–Whitney U-test was also used to compare the frequency of positive CD4<sup>+</sup> T-cell responses to deamidated and native gliadin during the follow-up (Figure 2A), the percentage of memory cells among proliferating CD4<sup>+</sup> T cells stimulated with deamidated or native gliadin (Figure 2B) and the frequency of  $\beta 7^{\text{high}}$  cells among dividing memory T cells stimulated with deamidated and native gliadin compared with tetanus toxoid (Figure 2C). Fisher's exact test was used to compare the cumulative incidences of tTGA seropositivity between girls and boys and to assess the frequency of CD4<sup>+</sup> T-cell responses to deamidated compared with native gliadin (Table I). *p*-Values of <0.05 were considered significant.

## Results

### Incidence of tTGA-seropositivity and celiac disease

Serum tissue transglutaminase IgA antibodies (tTGA) were determined at all sampling points starting from 12 months of age until the age of 36 or 48 months, and also at the age of 9 months in 41 children. Altogether, 19 children turned seropositive for tTGA (6.5%) during the follow-up (Figure 1). The median age at seroconversion was 24 months (range 18–48 months). Ten of the tTGA-positive children underwent duodenal biopsy and nine of these were diagnosed with CD (90%). The median age at the time of diagnosis was 3.5 years (2.6–4.2 years). In eight of the nine (88.9%) children with CD, the positive tTGA result in the follow-up was the initial finding resulting in further serological screening and clinical examination. Seven of these children subsequently developed gastrointestinal symptoms, such as runny stools, abdominal distension and abdominal pain before the duodenal biopsy, and only one of the diagnosed children was completely asymptomatic. Only one of the nine children with CD was examined due to the clinical suspicion of CD outside the study protocol. This child was tTGA seronegative at the age of 24 months but was later symptomatic and turned tTGA seropositive. CD was confirmed with the duodenal biopsy just before the age of 36 months. The cumulative frequency of tTGA seropositivity and the age at diagnosis of CD are shown in Figure 3. The one child with negative duodenal biopsy turned tTGA seronegative during the follow-up. Nine of the tTGA-seropositive children were not biopsied and seven of them had transient tTGA seropositivity, as they converted back to tTGA seronegative during the follow-up. In two children, no further information was available and for clarity, we have excluded these subjects from further analyses of the group of transient tTGA-seropositive children (a total of 291, 2.7%). The median level of tTGA at the time of seroconversion was significantly lower in the children not biopsied and/or turned

Table II. Development of anti-DGP antibodies before tTGA seroconversion.

Anti-DGP antibody positivity	Transiently tTGA seropositive ( <i>n</i> = 8)		Months before; median (range)	tTGA seropositive with confirmed CD ( <i>n</i> = 9)		Months before; median (range)
	IgA	IgG		IgA	IgG	
At tTGA seroconversion	0	5		9	9	
At least once before tTGA seropositivity	0	4	14 (6–24)	1	5	12 (6–18)
≥2 times before tTGA seropositivity	0	2	17 (9–24)	0	0	

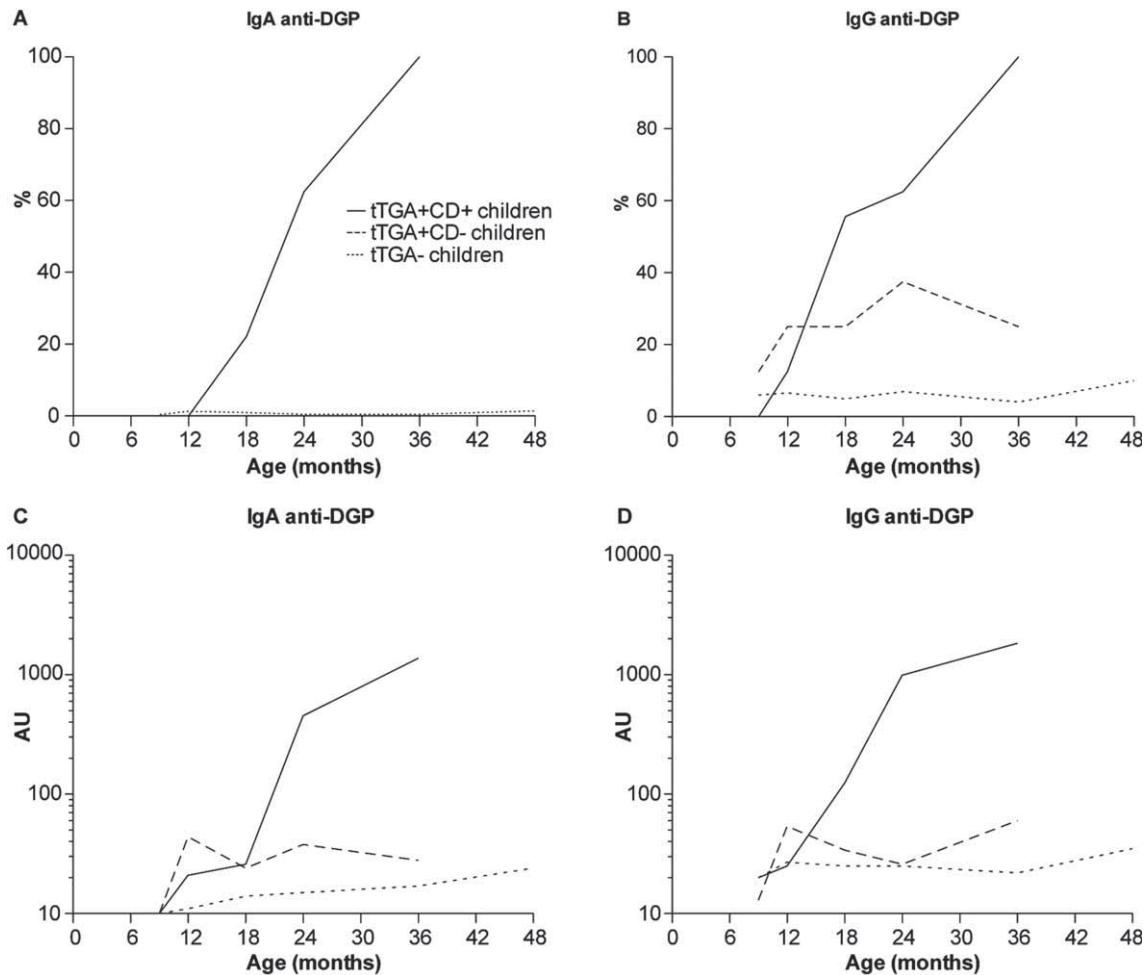


Figure 4. The frequency of IgA (A) and IgG (B) anti-DGP seropositivity and median levels of IgA (C) and IgG (D) anti-DGP antibodies at different sampling points in children with transient tTGA seropositivity (tTGA + CD-), in tTGA-positive children with confirmed CD (tTGA + CD+) and in children who remained tTGA seronegative (tTGA-) throughout the follow-up.

seronegative than in children with biopsy-proven CD ( $p = 0.004$ , median 9.6 and 100.0 IU, respectively, Mann-Whitney U-test). The cumulative incidence of tTGA seropositivity was significantly higher in girls than in boys (12.5% and 0.68%, respectively,  $p < 0.0001$ , Fisher's exact test) and all of the children diagnosed with CD were girls.

#### Development of anti-DGP antibodies

All except one of the children (18 of 19, 91.7%) who converted tTGA seropositive were also positive for anti-DGP antibodies at least at one sampling point using the earlier determined cut-off values 153 IU for IgA and 119 IU for IgG class anti-DGP antibodies [12]. All of the tTGA-seropositive children with confirmed CD (9 of 9, 100%) were both IgA and IgG anti-DGP positive at the time point of tTGA seroconversion. In contrast, only five of the eight children

(62.5%) with transient tTGA seropositivity were anti-DGP IgG positive at tTGA seroconversion and none of these children were positive for IgA anti-DGP (Table II).

Among children who remained tTGA seronegative during the follow-up, some developed IgA or IgG class anti-DGP. The percentage of IgA and IgG anti-DGP positivity in tTGA seronegative children during the follow-up varied between 0.4–1.4% and 4.1–10.0% at different time points, respectively (Figure 4A and B). Most of the tTGA seronegative children who developed anti-DGP positivity during the follow-up were positive only at a single sampling point and turned seronegative in the subsequent sample (36 of 272, 13.2%). Only 20 of 272 children (7.4%) were repeatedly IgA or IgG anti-DGP positive (at least in two sampling points), and half of them (10 of 272, 3.7%) were IgA or IgG anti-DGP positive at the last sampling point. The median levels of

anti-DGP antibodies in children with transient tTGA seropositivity, in tTGA-positive children who developed CD and in children remaining tTGA-seronegative are shown in Figure 4C and D.

*IgG-class anti-DGP antibodies precede tTGA seroconversion in children with CD*

In the group of tTGA-seropositive children with confirmed CD IgG anti-DGP positivity preceded tTGA seroconversion in over half of the cases (5 of 9, 55.6%) a median 12 months earlier (Table II). In one of the CD children also IgA anti-DGP antibodies appeared 12 months earlier than tTGA-seropositivity. Similarly, in the group of children with transient tTGA seropositivity IgG anti-DGP preceded tTGA seroconversion in 4 of the 8 subjects (50.0%) a median 14 months earlier (Table II). Two of the children with transient tTGA seropositivity were IgG anti-DGP positive at two previous sampling points before tTGA seroconversion (Table II).

*T-cell responses to deamidated and native gliadin*

Peripheral blood antigen-specific CD4<sup>+</sup> T-cell responses were studied in 85 children (39 girls and 46 boys) using a sensitive CFSE dilution-based *in vitro* proliferation assay (Table I). A total of 309 samples were studied. The frequency of T-cell responses to deamidated gliadin (gTG) was higher than that to native gliadin at all time points (Table I and Figure 2A). The highest frequency of positive responses to gTG was 40.5% (17 of 42) at the age of 9 months, and thereafter the percentage of positive gTG responses decreased during the course of sampling to the level of 22.2% (10 of 45) at the age of 48 months (Table I and Figure 2A). The frequency of positive responses to native gliadin declined even more compared with gTG, from the level of 20% to the level of 7–9% already at the age of 24 months (Table I and Figure 2A).

Memory and naïve CD4<sup>+</sup> T cells in the peripheral blood can be distinguished by their mutually exclusive expression of the CD45RO and CD45RA isoforms, respectively. We analyzed the expression of CD45RA and CD45RO molecules to determine the frequency of memory phenotype T cells (CD45RA-CD45RO<sup>+</sup>) within the proliferating antigen-specific CD4<sup>+</sup> T cells, as previously described [17]. The percentage of CD45RA-CD45RO<sup>+</sup> cells among proliferating CD4<sup>+</sup> T cells stimulated with deamidated gliadin and native gliadin did not differ. The percentage of CD45RA-CD45RO<sup>+</sup> cells among proliferating gTG-stimulated CD4<sup>+</sup> T cells was significantly higher at the last sampling point, 48 months of age, than at

9 months, demonstrating an increase in the frequency of responding memory CD4<sup>+</sup> T cells with age (median 81% and 92%, respectively,  $p = 0.003$ , Mann–Whitney U-test). The expression of  $\beta 7$ -integrin, a gut-homing molecule, was also analyzed in proliferating memory CD4<sup>+</sup> T cells [17]. The frequency of  $\beta 7^{\text{high}}$  cells was higher among the dividing CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells stimulated with both gTG (median 45.3%) and native gliadin (46.3%) compared with the memory T cells stimulated with tetanus toxoid (median 29.7%) ( $p < 0.0001$  and  $p = 0.0002$ , respectively, Mann–Whitney U-test) (Figure 2C). The higher expression of  $\beta 7$ -integrin demonstrates that memory CD4<sup>+</sup> T cells specific to both deamidated and native gliadin home to the gut where they have presumably been primed.

## Discussion

In this study, our aim was to analyze the development of gliadin-specific immune responses in children with a high genetic risk for CD and to determine whether these could be detected before the clinical onset of the disease by using immunological tests.

The cumulative incidence of tTGA seropositivity by the age of 4 years was 6.5%, which is in accordance with earlier studies where tTGA seropositivity in genetically predisposed children has been reported to vary between 2.6% and 6.0% by the age of 5–7 years [23–25]. The cumulative incidence of confirmed CD was 3.1%. All except one of the children with biopsy-confirmed CD were diagnosed after tTGA seropositivity during the follow-up without previous clinical suspicion. There were three children who seroconverted tTGA-positive at the age of 18 months. Interestingly, eight children with transient tTGA seropositivity, one of which also had a negative biopsy, had significantly lower tTGA titers at the time of seroconversion than the nine children later confirmed with CD. In a large Finnish DIPP follow-up study, the earliest seropositivity to tTGA has been reported to be at the age of 12 months [24]. Since in that study only 4 out of the 1320 children studied turned tTGA-seropositive that early, it seems that tTGA-seropositivity in children younger than 2 years is extremely rare. As described above, 8 of all 291 children (2.7%) studied here had transient tTGA-seropositivity and they turned tTGA-seronegative during the follow-up. Although it is known that clinically insignificant transient tTGA-seropositivity may occur in small children without gluten-free diet, fluctuating tTGA seropositivity may in some cases also precede the development of CD at later stage [24].



Several studies have demonstrated that anti-DGP antibodies perform with both high sensitivity and specificity in the diagnosis of CD both in adults and children [26–28]. Analogously, we have demonstrated earlier that our TR-IFMA anti-DGP assay performs with high sensitivity and specificity in pediatric patients [12]. Also in this prospective study, all of the tTGA seroconverted children with confirmed CD were both IgA and IgG anti-DGP positive at the time of tTGA seroconversion (Table II). Over half of the children with transient tTGA seropositivity were also IgG anti-DGP positive at the time of tTGA seroconversion but none of them were IgA anti-DGP positive, demonstrating the high specificity of IgA class anti-DGP assay for CD, as we have observed also earlier [12].

Not only tTGA seroconverted children, but also some of the children who remained tTGA seronegative developed anti-DGP antibodies. Altogether 76 of 1151 serum samples analyzed from children who remained tTGA seronegative during the follow-up were positive for IgG and only 17 for IgA anti-DGP, corresponding to calculated specificities of 93.4% and 98.5% for IgG and IgA anti-DGP, respectively. Since the follow-up time was limited we do not know whether some of the tTGA seronegative children repeatedly positive for anti-DGP will develop CD at a later stage. We can expect that the incidence of CD in these children at high genetic risk will remain at the same level than was the incidence during the follow-up [5,24]. A full evaluation of the significance of anti-DGP seropositivity would require a lengthy follow-up until adulthood.

IgA class anti-DGP positivity preceded tTGA seroconversion only in one child with confirmed CD, whereas in over half of the children with confirmed CD IgG class anti-DGP antibodies appeared a median 12 months earlier than tTGA seroconversion (Table II). The results of our prospective study concur with those of previous retrospective studies [11,12], which demonstrate that IgG anti-DGP seropositivity precedes tTGA-seropositivity. However, in the current study a similar phenomenon was observed in children with transient tTGA seropositivity. Therefore, IgG anti-DGP antibodies have a good sensitivity to predict tTGA seropositivity but appear not to clearly discriminate between CD-associated and transient tTGA seropositivity, potentially hindering the clinical usefulness of the phenomenon.

In the present study, we also followed prospectively the development of peripheral blood gliadin-specific T-cell responses in early childhood. We have demonstrated previously that CD4<sup>+</sup> T cells specific to deamidated gliadin can be detected at a higher frequency in the peripheral blood of pediatric patients

at the time of CD diagnosis [17]. Liu et al. have also demonstrated that gliadin-specific T-cell responses can be detected without gluten challenge in peripheral blood of children with CD [18]. However, there are no prospective follow-up studies of CD where gliadin-specific T-cell responses have been monitored in children. Here, altogether 85 children were studied for T-cell responses by using a sensitive CFSE-dilution method [17,22]. T-cell responses to deamidated gliadin were detected at a higher frequency than those to native gliadin at all time points in HLA-DQ2-positive children, as reported also previously in children with CD [17]. Our previous and current results are consistent with the idea that deamidation of gliadin epitopes enhances their T-cell reactivity in the context of HLA-DQ2 [13]. Interestingly, the highest frequency of T-cell responses to both deamidated gliadin and native gliadin was observed at the age of 9 months and the frequency decreased during the follow-up. The frequency of gTG-specific T-cell responses at the age of 48 months (22.2%) was similar to what we have previously reported (22.4%) in healthy children with HLA-associated genetic risk for CD [17]. The high rate of T-cell responses both to deamidated and native gliadin in the earliest samples could be associated with early immunization to dietary gluten, which in most cases does not result in an antibody response to deamidated gliadin. Of note, IgG class antibody responses to whole gliadin are also commonly observed in young children, and they are not always associated with the development of CD [29,30], supporting the concept that not all observed immune responses to dietary gluten are pathogenic. We did not directly test whether the observed gTG-specific T-cell responses were from memory origin or were restricted by HLA-DQ2. However, staining for CD45RA and CD45RO suggested that in children positive for HLA-DQ2 CD4<sup>+</sup> T cells specific to deamidated and native gliadin have mainly a memory phenotype. No differences in the frequency of memory CD4<sup>+</sup> T cells were observed between gTG and native gliadin-stimulated cultures, which are in accordance with our earlier results in healthy controls carrying the CD-related HLA-risk alleles [17]. The percentage of CD45RA-CD45RO<sup>+</sup> memory T cells in gTG-stimulated cultures was highest at the last sampling point, supporting the hypothesis the immune response to gTG matures with age. The expression of the gut-homing  $\beta$ 7-integrin was significantly higher among proliferating memory T cells stimulated with either gTG or native gliadin than those stimulated with tetanus toxoid. No differences in the expression of  $\beta$ 7-integrin were detected between proliferating memory T cells stimulated with gTG and native

gliadin, which is also in line with the earlier results demonstrated in healthy controls [17]. These findings support the hypothesis that the CD4<sup>+</sup> T cells specific to gliadin are generated in the intestinal mucosa and are capable of homing back to the intestine after circulating in peripheral blood. Based on our current data, we cannot ascertain whether the emergence of gliadin-specific T-cell responses is associated with the introduction of gluten to diet since we did not analyze blood samples taken before 9 months of age. Taken together, our results indicate that peripheral blood gliadin-specific T-cell responses are relatively common in HLA-DQ2-positive children and appear not to be directly associated with the development of CD. Oral tolerance to dietary antigens develops early in life, and CD can be comprised either as a failure to develop oral tolerance to gluten or as a later loss of the tolerance [31]. Peripheral blood T-cell responses to gliadin and gTG may therefore probably reflect the early immunization and subsequent development of T-cell tolerance to dietary gluten at early infancy.

In summary, we have followed prospectively the emergence of both tTGA and anti-DGP seropositivity in a cohort of children with a high genetic risk for CD until the age of 4 years. Most of the children who developed CD during the follow-up were diagnosed due to a positive tTGA-result in the serological screening without previous clinical suspicion. In line with previous retrospective studies, we demonstrate that anti-DGP antibodies may precede tTGA seroconversion and thus may allow earlier detection of CD. We also followed prospectively circulating T-cell responses to deamidated gliadin and demonstrate for the first time that these responses are very common already at an early age of 12 months in HLA-DQ2-positive children.

### Acknowledgements

The authors thank Anne Björk and Sirpa Nolvi for carrying out the recruitment as well as the organization of sample collection with the participating families. The authors also thank Virpi Fisk and Terttu Laurén for excellent technical assistance. This study was financially supported by grants from the Finnish Coeliac Society, The Finnish-Norwegian Medical Foundation, The Finnish Cultural Foundation, Päivikki and Sakari Sohlberg Foundation, Finland and Special Research Funds for Kuopio University Hospital.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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