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RANDOMIZED FEEDING INTERVENTION IN INFANTS AT HIGH RISK FOR COELIAC DISEASE

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ABSTRACT

Background

A window of opportunity has been suggested for reducing the risk of coeliac disease by introducing gluten to infants at 4 to 6 months of age.

Methods

We performed a multicenter, randomized, double-blind, placebo-controlled dietary intervention study involving 944 children who were positive for HLA-DQ2 or HLA-DQ8 and had at least one first-degree relative with coeliac disease. From 16 to 24 weeks of age, 475 participants received 100 mg of immunologically active gluten daily, and 469 received placebo. Anti–transglutaminase type 2 and antigliadin antibodies were periodically measured. The primary outcome was the frequency of biopsy-confirmed coeliac disease at 3 years of age.

Results

Coeliac disease was confirmed by means of biopsies in 77 children. To avoid underestimation of the frequency of coeliac disease, 3 additional children who received a diagnosis of coeliac disease according to the 2012 European Society for Pediatric Gastroenterology, Hepatology, and Nutrition diagnostic criteria (without having undergone biopsies) were included in the analyses (80 children; median age, 2.8 years; 59% were girls). The cumulative incidence of coeliac disease among patients 3 years of age was 5.2% (95% confidence interval [CI], 3.6 to 6.8), with similar rates in the gluten group and the placebo group (5.9% [95% CI, 3.7 to 8.1] and 4.5% [95% CI, 2.5 to 6.5], respectively; hazard ratio in the gluten group, 1.23; 95% CI, 0.79 to 1.91). Rates of elevated levels of anti–transglutaminase type 2 and antigliadin antibodies were also similar in the two study groups (7.0% [95% CI, 4.7 to 9.4] in the gluten group and 5.7% [95% CI, 3.5 to 7.9] in the placebo group; hazard ratio, 1.14; 95% CI, 0.76 to 1.73). Breast-feeding, regardless of whether it was exclusive or whether it was ongoing during gluten introduction, did not significantly influence the development of coeliac disease or the effect of the intervention.

Conclusions

As compared with placebo, the introduction of small quantities of gluten at 16 to 24 weeks of age did not reduce the risk of coeliac disease by 3 years of age in this group of high-risk children. (Funded by the European Commission and others; PreventCD Current Controlled Trials number, ISRCTN74582487)

INTRODUCTION

Coeliac disease (CD), an immune-mediated systemic disorder elicited by gluten in genetically susceptible persons, is characterized by anti-transglutaminase type 2 antibodies (TG2A) and enteropathy.[1] The prevalence of CD is 1-3% in the general population and approximately 10% among first-degree family members of patients with CD.[2-10] CD is treated with a gluten-free diet. More than 95% of patients have the HLA-DQ2 heterodimer, either in the *cis* or *trans* configuration. Most of the remaining patients have the HLA-DQ8 heterodimer or half of the DQ2 heterodimer (DQB1*02).[1, 8, 11-14] However, more than 25% of the general population carries these haplotypes,[8, 13] indicating that additional factors are involved in disease development. CD increases overall mortality risk,[15] reduces quality of life,[16] and has extensive negative economic consequences.[17, 18] The health and quality of life of patients improves with a gluten-free diet, but primary prevention would be more beneficial.[19, 20] Results from observational studies indicate that the development of oral tolerance for gluten is initiated early in life, and that the mode of introducing gluten to infants may influence the risk of CD in predisposed persons.[21-25] The results of these studies suggest that there is a "window of opportunity" at 4 to 6 months of age, when the first exposure to gluten should occur in order to decrease the risk of CD.[24, 25] The results of studies evaluating breastfeeding and the risk for CD are inconclusive, since most of these studies were retrospective and associated with parental recall bias, and none included randomization or specified the quantities of gluten consumed.[23-27] At present, the true influence of early feeding on the development of CD remains controversial.

To investigate the possible primary prevention of CD, the European multicenter project "Prevent Coeliac Disease" (PreventCD; www.preventcd.com) was initiated.[19] It was hypothesized that the frequency of CD at 3 years of age could be reduced by exposing genetically predisposed infants to small quantities of gluten at 16 to 24 weeks of age, preferably while they were still being breastfed.

METHODS

Study design and participants

We performed a prospective, randomized, double-blind, placebo-controlled, dietary-intervention study. The first child was included on May 26, 2007, and the follow-up for this analysis closed on September 10, 2013, when the youngest study participant turned 3 years of age; the oldest participants were up to 6 years of age.

Infants 0 to 3 months of age were recruited consecutively through CD organizations from Croatia, Germany, Hungary, Israel, Italy, the Netherlands, Poland, and Spain. Infants were required to have the HLA-DQ2, HLA-DQ8, or HLA-DQB1*02 heterodimer (centrally typed) and to have at least one first-degree family member with CD confirmed by means of small-bowel biopsies. We excluded premature infants and those with trisomy 21 and Turner's syndrome (online supplementary appendix, available at nejm.org).

Intervention

We randomly assigned participants to receive either 200 mg of vital wheat gluten mixed with 1.8 g lactose (equivalent to 100 mg of immunologically active gluten), or to placebo (2 g lactose), given daily for 8 weeks starting at 16 weeks of age (online supplementary appendix). Previous assessment of the vital wheat gluten by means of ELISA and Western blot analysis had shown the presence of gluten proteins typically found in wheat gluten. Randomization, stratified by participating country, was performed with the use of variable block sizes ranging from 4 to 8 and with SPSS software (version 18.0). The investigators and the parents of the participants were unaware of the intervention assignments. Adherence to the study assignment was assessed by means of frequent interviews with the parents (online table S1). Participants were considered to have adhered to the intervention assignment if at least 75% of the material was ingested and no additional gluten was consumed. After the intervention, parents were advised to introduce gluten gradually, using regular products and standardized recommendations (online supplementary appendix).

Outcomes

The primary outcome was the frequency of CD at 3 years of age. The diagnosis of CD was based on the histologic findings of small-bowel biopsies, according to the 1990 criteria of the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN).[28] Secondary end points were the occurrence of symptoms and the immune response to gluten as indicated by elevated serum antibodies associated with CD (anti-gliadin antibodies and TG2A) (online supplementary appendix).

Follow-up and assessment of CD

We periodically monitored health status, anthropometric variables, and feeding habits (i.e. breastfeeding and formula feeding), and we quantified gluten consumption[29] using standardized questionnaires (online table S1). Measurement of serum antigliadin and TG2A were performed centrally at least seven times during the first 3 years of age and then annually thereafter. The parents of children with elevated CD-associated antibodies or with symptoms suggesting CD were offered small-bowel biopsies to confirm the diagnosis in their child (online supplementary appendix). The biopsy specimens were histologically assessed at the study sites and were also reviewed by an author who is a pathologist.[30] The

age of the patient at which the diagnostic biopsies were performed was considered to be the age at the diagnosis of CD.

Study oversight

The study was approved by the medical ethics committee at each participating center and complied with the Good Clinical Practices regulations (online supplementary appendix). The authors vouch for the veracity and completeness of the data and analyses reported and for the adherence of the study to the protocol, available at nejm.org.

From 2007 to 2011, the study did not have commercial support. After 2011, Thermo Fisher Scientific performed antibody assessments without charge, and together with Eurospital and Fria Bröd, Thermo Fisher Scientific partly funded the project progress meetings. The funding organizations had no role in the conception, design, or conduct of the study, in the analysis or interpretation of the data, or in the writing of the manuscript of the decision to submit it for publication.

Statistical analysis

To detect a 50% reduction in the development of CD in the gluten group at 3 years of age (5%, versus 10% with placebo) with a two-sided significance level of 5% and with 80% power, we calculated that 474 children would be required in each group.[19]

All the data were entered into a Web-based data management application with the use of a central structured-query-language server database (NEN 7510 certified). A statistical analysis plan was published online before the randomization codes were opened (http://preventcd.com/images/stories/Publications/PreventCD_SAP_1_0.pdf, online supplementary appendix). For estimating the cumulative incidence of CD, Kaplan-Meier curves were calculated, with time defined as the patient's age at diagnosis of CD or at the last assessment or withdrawal from the study (when data were censored). For comparison, a log-rank test (two-sided) was used, stratified according to participating country. The hazard ratio for CD in the gluten group, as compared with the placebo group (with 95% confidence intervals), is provided, on the basis of a Cox proportional-hazards regression analysis. The primary analysis was performed according to the intention-to-treat principle. Differences in cumulative incidence of CD were assessed according to the baseline variables by means of Cox proportional-hazards regression (multivariate) analysis and according to the duration of breast-feeding, daily gluten intake, and occurrence of infection by means of a landmark analysis (online supplementary appendix). Different intervention effects were assessed in subgroups by including an interaction term between intervention and subgroup in the Cox proportional-hazards regression analysis. Analyses were performed with SPSS software (version 20.0).

RESULTS

Characteristics of the participants

The parents of 1343 children provided written informed consent for the study. A total of 963 children were randomly assigned to receive gluten (483 participants) or placebo (480) (Figure 1 and online supplementary appendix). After randomization, the number of children was reduced to 944 because 19 children did not fulfill the inclusion criteria. A total of 99 children (10.5%) did not adhere to the intervention assignment (59 children in the gluten group and 40 in the placebo group). A total of 141 children stopped participating before 3 years of age (withdrawal rate 14.9%, 69 participants in the gluten group and 72 in the placebo group). A total of 59 children withdrew during the first year (6.2%), 49 during the second year (5.2%), and 33 during the third year (3.5%); the median follow-up was 4 years (range, 22 days to 6.30 years). The reasons for withdrawal were unknown for 57% of the children, were related to practical issues for 39% (e.g. blood sampling or travel distance to center), and were related to adverse events for 4% (online supplementary appendix).
The baseline characteristics of the children were similarly distributed between the intervention groups, with the exception of homozygosity for HLA-DQ2 (Table 1). Data on breast-feeding were available for 943 children: 882 started breast-feeding; at 6 months of age, 527 (55.8%) were breast-fed, and 265 (28.1%) were breast-fed without complementary feeding except for the intervention product. Of the 455 mothers with CD, 431 were consuming a gluten-free diet during pregnancy and lactation. Rotavirus vaccination was performed in 211 children (22.4%), either before the intervention (176 children) or during the intervention (35).

Diagnosis of CD

The numbers of children who met the criteria to undergo small-bowel biopsies are shown in Figure 1. A total of 101 small-bowel biopsies were performed in 94 children (Table 2, and online supplementary appendix). CD was confirmed by means of biopsies in 77 children. To avoid underestimation of the frequency of CD, 3 additional children, whose parents declined biopsies on behalf of their children but who complied with the 2012 ESPGHAN diagnostic criteria,[1] were considered to have CD in all analyses (Figure 1).

The median age of the 80 children at diagnosis was 2.8 years (range, 1.1 to 5.6), and all the children had an elevated level of TG2A; 59% were girls. The most frequent symptoms were abdominal distension (in 20 children) and diarrhea (in 19). The cumulative incidence of CD at 3, 4 and 5 years of age was 5.2% (95% confidence interval [CI] 3.6 to 6.8), 8.8% (95% CI 6.6 to 11.0) and 12.1% (95% CI 9.2 to 15.0) respectively (online table S2, online figure S1). CD was significantly more frequent in girls; at 3 years of age, the cumulative incidence among girls and boys was 7.2% and 3.4%, respectively; at 4 years of age, 11.8% and 6.1%, and at 5 years of age, 14.5% and 9.9% (p=0.04 by the log-rank test, p=0.02 by multivariate analysis) (online table S2).

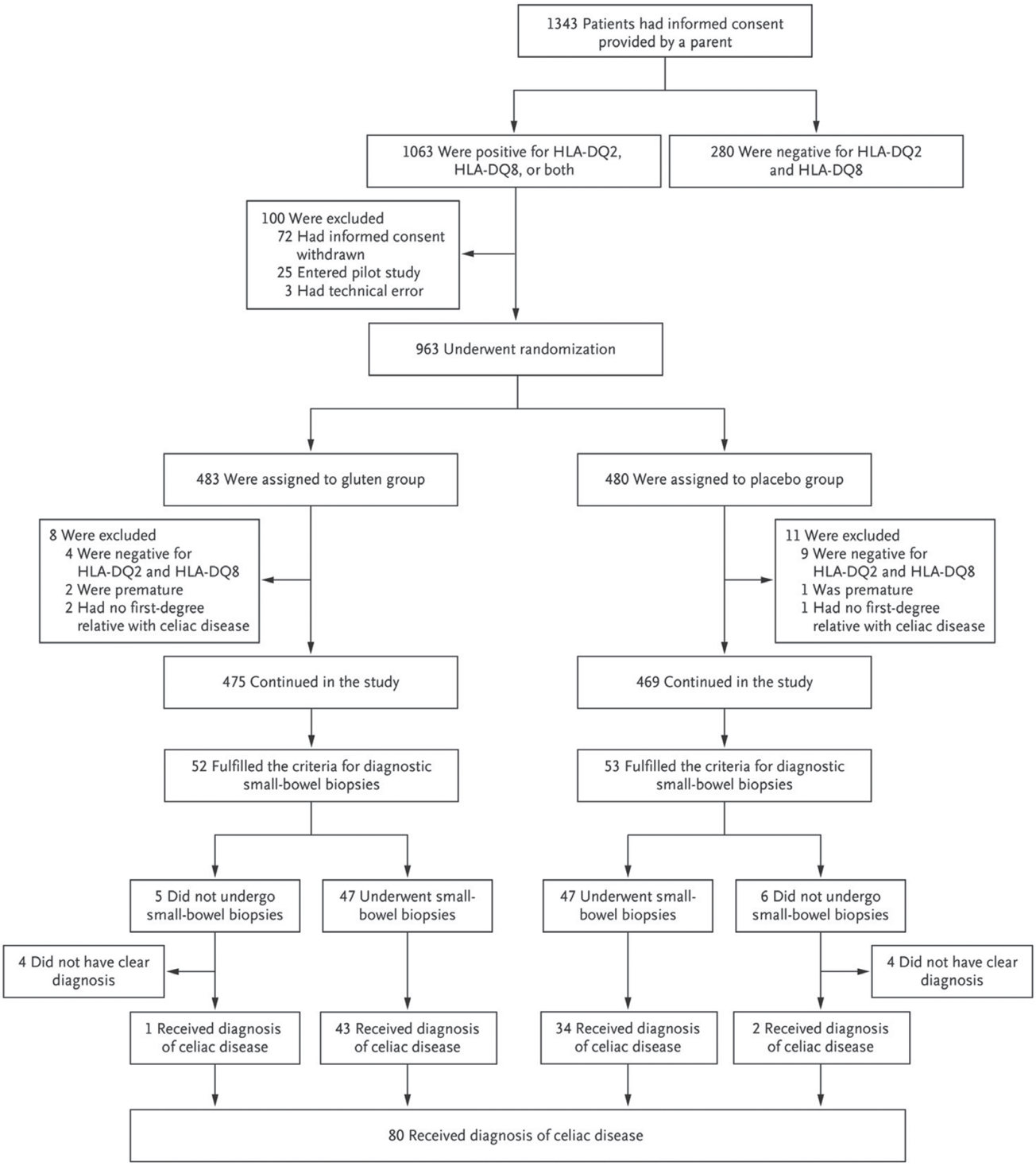

Figure 1 Randomization and diagnosed cases of coeliac disease. A total of 25 children were included in a pilot study to test the infrastructure of the study and were not included in the primary analysis. A total of 19 children underwent randomization in error and were excluded from the study. On the basis of histologic results of small-bowel biopsies, active CD was ruled out in 17 children, although 3 of the 17 had potential CD. There was no clear diagnosis in 8 asymptomatic children whose parents declined small-bowel biopsies on their behalf and who had transient levels of CD–associated antibodies. CD was diagnosed in 3 children according to the 2012 European Society for Pediatric Gastroenterology, Hepatology, and Nutrition diagnostic criteria (without having undergone biopsies).[1]

Table 1 Characteristics of the participating children.[a]

		Gluten (N=475)	Placebo (N=469)
Age (in years) at end of follow-up for this analysis, mean (min-max)		4.9 (3.1-6.5)	5.0 (3.1-6.6)
Female sex, no. (%)		228 (48.0)	226 (48.2)
Gestational age (in weeks), mean (min-max)		39.1 (34-43)	39.2 (35-42)
Birth weight (in grams), mean (min-max)		3316 (1730[b]-5000)	3346 (2000-4740)
Country, no. (%)	Spain	130 (27.4)	119 (25.4)
	Italy	70 (14.7)	69 (14.7)
	Hungary	70 (14.7)	68 (14.5)
	The Netherlands	67 (14.1)	66 (14.1)
	Germany	55 (11.6)	58 (12.4)
	Israel	47 (9.9)	48 (10.2)
	Poland	30 (6.3)	34 (7.2)
	Croatia	6 (1.3)	7 (1.5)
HLA-risk group[c], no./total no. (%)	1	80/462 (17.3)	49/449 (10.9)
	2	46/462 (10.0)	42/449 (9.4)
	3	199/462 (43.1)	218/449 (48.6)
	4	29/462 (6.3)	37/449 (8.2)
	5	108/462 (23.4)	103/449 (22.9)
First degree relatives with CD, no. (%)	1	431 (90.7)	432 (92.1)
	2	42 (8.8)	32 (6.8)
	3 or more	2 (0.4)	5 (1.1)
Type of first degree relative with CD, no. (%)	Mother only	200 (42.1)	207 (44.1)
	1 sibling	183 (38.5)	184 (39.2)
	Father only	48 (10.1)	41 (8.7)
	Mother and ≥1 sibling	23 (4.8)	23 (4.9)
	>1 sibling, but neither parent	12 (2.5)	7 (1.5)
	Father and ≥1 sibling	9 (1.9)	5 (1.1)
	Mother + father	0	2 (0.4)

[a] The characteristics of the children were similarly distributed between the intervention groups ($P < 0.05$), with the exception of homozygosity for HLA-DQ2 ($P = 0.05$).

[b] Data included a pair of healthy twins.

[c] Data on the HLA-risk groups were available for 911 of 944 children, with HLA typing performed by means of single-nucleotide polymorphisms (SNPs) on the basis of the tag-SNP approach.[8] The HLA risk groups were defined as follows: group 1 included DR3–DQ2/DR3–DQ2 (DQ2.5/DQ2.5) and DR3–DQ2/DR7–DQ2 (DQ2.5/DQ2.2); group 2 DR7–DQ2/DR5–DQ7 (DQ2.2/DQ7); group 3 DR3–DQ2/DR5–DQ7 (DQ2.5/DQ7), DR3–DQ2/DR4-DQ8 (DQ2.5/DQ8), and DR3–DQ2/other (DQ2.5/other); group 4 DR7–DQ2/DR7–DQ2 (DQ2.2/DQ2.2), DR7–DQ2/DR4–DQ8 (DQ2.2/DQ8), and DR4–DQ8/DR4–DQ8 (DQ8/DQ8); and group 5 DR7–DQ2/other (DQ2.2/other), DR4–DQ8/DR5-DQ7 (DQ8/DQ7), and DR4–DQ8/other (DQ8/other); "other" refers to any HLA-DQ haplotype except DR3–DQ2, DR7–DQ2, DR4–DQ8, or DR5–DQ7. For the remaining 33 children, the status with regard to HLA-DQ2 and HLA-DQ8 positivity was determined by means of the EU-Gen Risk test (Eurospital), with no information provided regarding the HLA risk group.

Table 2 Distribution of symptoms and coeliac disease (CD) associated antibodies in 94 children with suspected CD, who underwent 101 diagnostic small-bowel biopsies.[a]

Variable		Eventual diagnosis				
		CD (77 biopsies)	Potential CD[b] (5 biopsies)	Unclear diagnosis (2 biopsies)	No CD (17 biopsies)	Total (101 biopsies)
Symptoms as indication for biopsy (no.)		52	0	2	13	67
Elevated TG2A level as indication for biopsy (no.)[c]		77	5	0	0	82
Elevated antigliadin antibodies as indication for biopsy (no.)***		12	0	0	6	18
Marsh classification of findings in small bowel biopsies (no.)[d]	0	0	4	0	13	17
	1	0	1	0	1	2
	2	3[e]	0	2	3	8
	3A	18	0	0	0	18
	3B	24	0	0	0	24
	3C	32	0	0	0	32
	4	0	0	0	0	0

[a] One child with an elevated anti-transglutaminase type 2 antibodies (TG2A) level underwent biopsy three times: the histologic findings were normal the first two times but compatible with CD the last time. Five children underwent small-bowel biopsies twice. The first time, all had normal histologic findings; the second time, two children had normal histologic findings (none had potential CD), and three received a diagnosis of CD.

[b] Potential CD was defined as an elevated level of TG2A and histologic findings in the small bowel.

[c] An elevated serum level of IgA TG2A was defined as a level of 6 U/ml or more (or in the case of IgA deficiency, an IgG TG2A level of ≥ 10 U/ml). An elevated anti-gliadin antibody level was defined as a level of more than 50 U/ml (or in the case of IgA deficiency, an IgG anti-gliadin level of ≥ 17 U/ml) on three occasions during a 3-month period, or a level of more than 17 U/ml that was clearly increasing in two tests performed during a 3-month period.

[d] Findings of small-bowel biopsies were assessed according to the Marsh classification,[30] on a scale from 0 to 4, with classes 0 and 1 being not characteristic of CD, class 2 being compatible with CD only with a concomitant elevated TG2A level, classes 3A to 3C being characteristic of CD (with higher letter grades indicating more villous atrophy), and class 4 being characteristic of refractory CD.

[e] Three children had a concomitant elevated TG2A level, as compared with the other five children with a Marsh classification of 2.[1]

The disease developed significantly more frequently and earlier in the group of children who were homozygous for HLA-DQ2 (DR3-DQ2/DR3-DQ2 or DR3-DQ2/DR7-DQ2) than in other HLA-risk groups,[4] with cumulative incidence at 3, 4, and 5 years of age of 14.9%, 23.9% and 26.9% respectively ($p<0.001$) (online table S2, online figure S2).

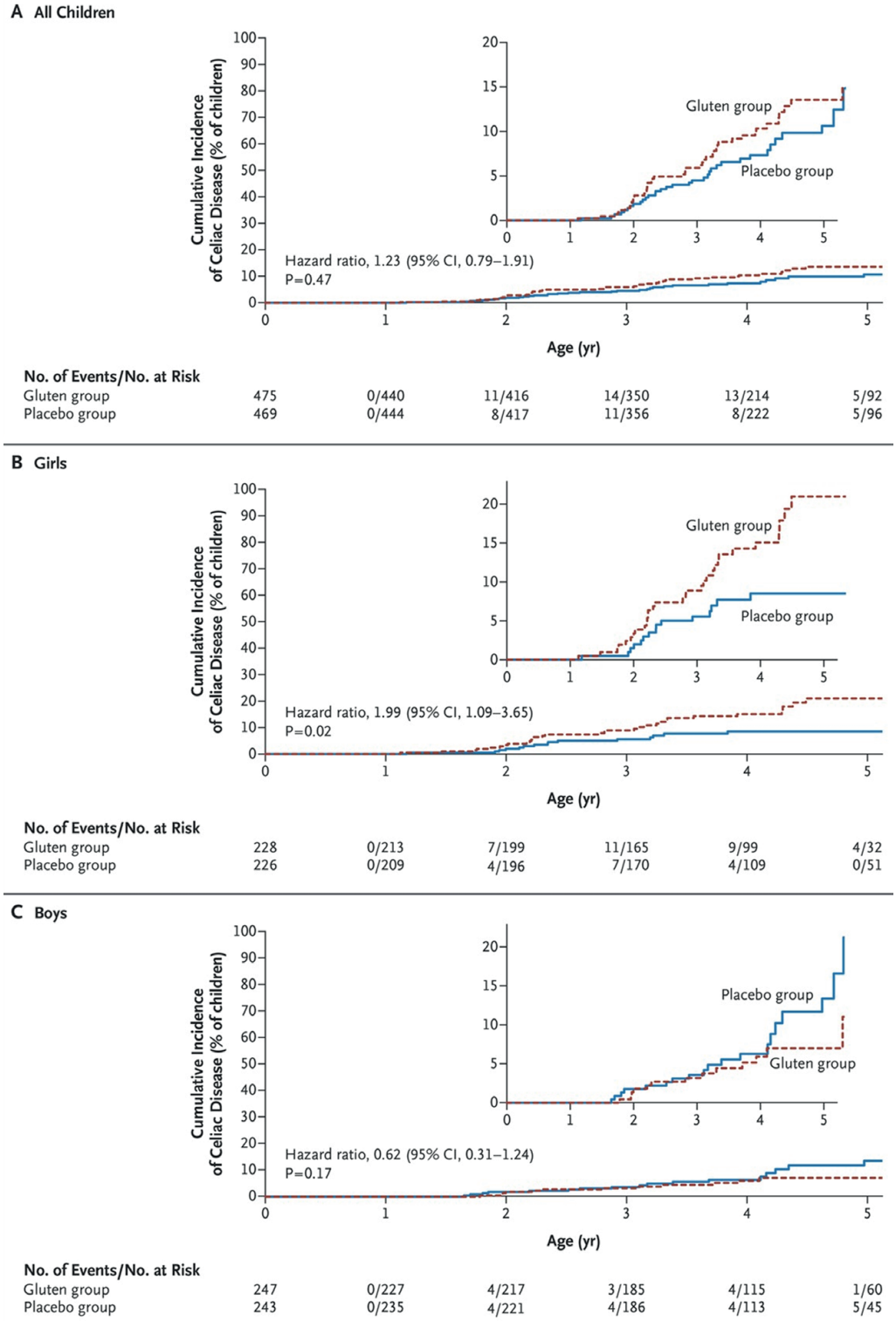

Figure 2 Cumulative incidence of coeliac disease (CD). A total of 75 of 80 children received a diagnosis of CD before 5 years of age. The cumulative incidence of CD in the gluten group versus the placebo group at 3, 4, and 5 years of age was as follows: 5.9% versus 4.5%, 10.3% versus 7.3%, and 13.5% versus 10.6%, respectively (Panel A). The cumulative incidence among 454 girls in the gluten group and the placebo group was as follows: 8.9% versus 5.5%, 15.1% versus 8.5%, and 21.0% versus 8.5%, respectively (Panel B). The cumulative incidence among 490 boys was as follows: 3.2% versus 3.6%, 5.9% versus 3.6%, and 7.0% versus 13.4%, respectively (Panel C). The data in Panels B and C show a significant interaction between sex and intervention (P=0.01). The insets show the same data on an expanded y axis.

Breast-feeding did not influence the development of CD. The cumulative incidence at 3 years of age among children who were not breast-fed, were breastfed for 3 or fewer months, were breast-fed for 4 or 5 months, or were breast-fed for 6 or more months were 7.3%, 4.4%, 8.2% and 4.4%, respectively (p=0.28). Similar cumulative incidences at 3 years of age were observed among children who were never exclusively breast-fed or were breast-fed exclusively for 3 months or less, for 4 or 5 months, and for 6 months or more (5.0%, 9.1%, 5.3% and 2.7%, respectively; p=0.45). Country of origin and the number and type of affected family members were also not related to the development of disease (online table S2), nor were rotavirus vaccination, gastrointestinal or respiratory tract infection, and mean daily gluten intake (online supplementary appendix).

Development of CD in relation to the intervention

The intervention with gluten, as compared to placebo, did not have a significant effect on the frequency of CD development, with cumulative incidences at 3 years of age of 5.9% (95% CI 3.7 to 8.1) and 4.5% (95% CI 2.5 to 6.5), respectively (p=0.47 by a stratified log-rank test; hazard ratio, 1.23; 95% CI 0.79 to 1.9) (Figure 2A). The duration of breast-feeding, whether exclusive or not, did not significantly influence the effect of the intervention on the development of CD (p=0.70 [for exclusive breast-feeding] and p=0.83 [for nonexclusive breast-feeding] for interaction; hazard ratios are provided in online table S3).

The cumulative incidence of CD was significantly higher in girls randomly assigned to gluten than among those randomly assigned to placebo: at 3 years of age, the incidence was 8.9% in the gluten group versus 5.5% in the placebo group (hazard ratio 1.99; 95% CI 1.09 to 3.65; p=0.02) (Figure 2B). This difference was not seen among boys, with frequencies of 3.2% in the gluten group and 3.6% in the placebo group (hazard ratio 0.62; 95% CI 0.31 to1.24; p=0.17; P=0.01 for interaction of sex and intervention) (Figure 2C). No other factors than sex were found to significantly influence the effect of the intervention on the development of CD (Figure 3, online table S3).

The results of the primary per-protocol analysis were similar to those of the intention-to-treat analysis (online supplementary appendix). The cumulative incidence of CD seropositivity (positive TG2A, positive anti-gliadin antibodies, or both on two occasions during a 3-month period) did not differ significantly between the gluten group and the placebo group (7.0% [95% CI, 4.7 to 9.4] and 5.7% [95% CI, 3.5 to 7.9], respectively; hazard ratio, 1.14 95% CI, 0.76 to 1.73; p=0.53) (Table 3, online figure S3). Although elevated levels of TG2A were not found in any of the participants at 6 months of age, transient anti-gliadin antibody levels of more than 17 U/ml were observed in 59 children in the gluten group and 2 in the placebo group. This elevation was not predictive of CD, which developed in only 8 of these children, all in the gluten group.

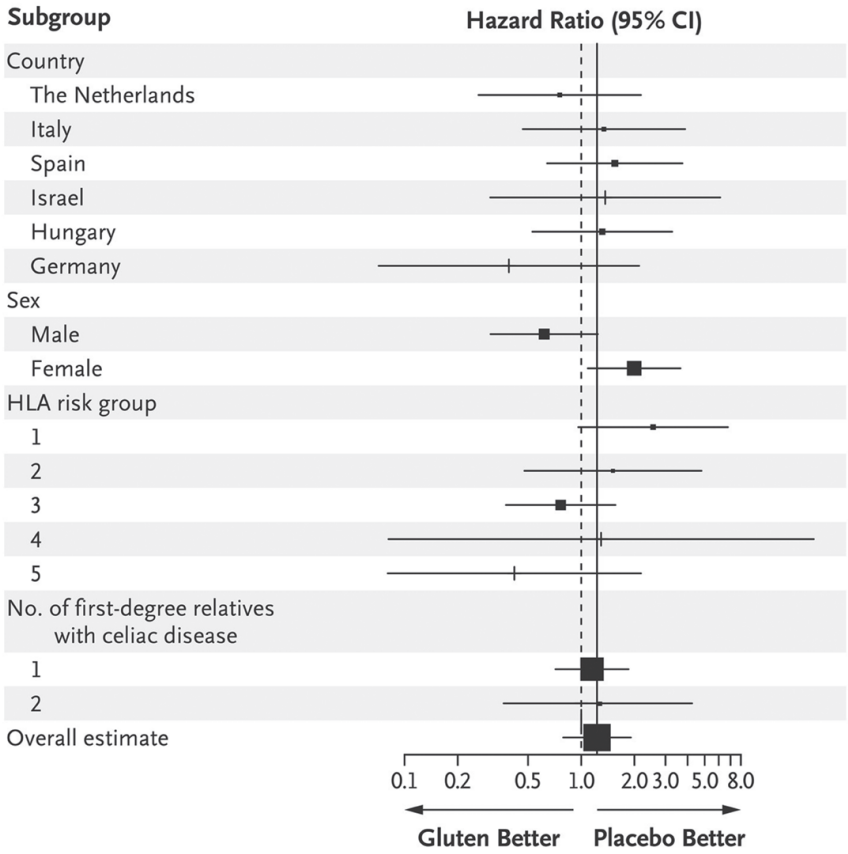

Figure 3 Effect of intervention assignment at 16 to 24 weeks of age on the development of coeliac disease (CD) in 944 children from high-risk families. Female sex was the only factor to significantly favor placebo (P=0.02). The HLA risk groups were defined as follows: group 1 included DR3–DQ2/DR3–DQ2 (DQ2.5/DQ2.5) and DR3–DQ2/DR7–DQ2 (DQ2.5/DQ2.2); group 2 DR7–DQ2/DR5–DQ7 (DQ2.2/DQ7); group 3 DR3–DQ2/DR5–DQ7 (DQ2.5/DQ7), DR3–DQ2/DR4-DQ8 (DQ2.5/DQ8), and DR3–DQ2/other (DQ2.5/other); group 4 DR7–DQ2/DR7–DQ2 (DQ2.2/DQ2.2), DR7–DQ2/DR4–DQ8 (DQ2.2/DQ8), and DR4–DQ8/DR4–DQ8 (DQ8/DQ8); and group 5 DR7–DQ2/other (DQ2.2/other), DR4–DQ8/DR5-DQ7 (DQ8/DQ7), and DR4–DQ8/other (DQ8/other); "other" refers to any HLA-DQ haplotype except DR3–DQ2, DR7–DQ2, DR4–DQ8, or DR5–DQ7. No statistics were computed for children from Poland (64 children) and Croatia (13), or for children with three or more first-degree relatives with CD (7) because of the low number of children with CD in these groups. The black boxes represent the hazard ratio with 95% confidence intervals (horizontal lines); the size of each box is proportional to the size of the corresponding subgroup. The overall estimate is represented by the solid vertical line; a dashed vertical line representing no effect is also shown.

DISCUSSION

Our results indicate that the early introduction (at 16 weeks of age) of small quantities of gluten did not reduce the risk of CD at 3 years of age in genetically predisposed children from high-risk families; therefore, our results do not support the protective effect that we had hypothesized. In addition, we show that breast-feeding, whether exclusive or not, did not have a significant effect on the frequency of CD among these children. In prespecified secondary analyses, we observed an association between the early gluten intervention

Table 3 Antibody elevations and diagnosis of coeliac disease (CD) according to intervention assignment.[a]

Variable		Cumulative incidence		P value[b]	Hazard ratio (95% CI)
		Gluten (N=475)	Placebo (N=469)		
		%			
Elevated anti-gliadin at age 6 months of age		12.4	0.4	<0.001	
Elevated TG2A at 6 months of age		0.0 (0)	0.0 (0)	NA	
Elevated level of TG2A or anti-gliadin antibody	At 1 yr of age	0.9	0.0	0.53	1.14 (0.76-1.73)
	At 2 yr of age	3.2	2.1		
	At 3 yr of age	7.0	5.7		
	At 4 yr of age	11.5	9.5		
	At 5 yr of age	14.0	12.1		
CD	At 1 yr of age	0.0	0.0	0.47	1.23 (0.79-1.91)
	At 2 yr of age	2.6	1.9		
	At 3 yr of age	5.9	4.5		
	At 4 yr of age	10.3	7.3		
	At 5 yr of age	13.5	10.6		

[a] NA denotes not applicable

[b] The p-value for the elevated antibody level at 6 months of age was calculated by means of a Fisher's exact test, and the other p-values were calculated by means of the log rank test.

and CD in girls but not in boys. We did not find significant effects in the other subgroups examined, and the significant finding in girls may be due to chance or to the larger number of girls with HLA-DQ2 homozygosity who were randomly assigned to gluten rather than to placebo (online table S4). Owing to the small number of children in the different HLA risk groups stratified according to sex, we cannot resolve this issue.

The higher frequency of CD among girls than among boys after early exposure to gluten may be related to the well-known increased risk of CD among women[13, 31] , but it appears too early in life to be related to the protective effect of androgens for autoimmunity.[32] The gut microbiota may also play a role in this sexual dimorphism, as was shown recently for type 1 diabetes in rodents, in which hormones and microbes together trigger protective pathways.[32, 33] Our results also show prospectively the effect of HLA-DQ2 homozygosity on the risk of CD in early childhood.

In general, we found no association between the early development of CD and the presence of the disease in one or both parents, but this finding should be interpreted with caution,

given the small number of fathers with CD in our cohort (105 of 944). Possible explanations for the small number of affected fathers are the tendency for mothers to be more involved in research projects[34] and the higher frequency of CD among women.[13]

Contrary to previous reports,[35, 36] our data show that determining the TG2A level, but not the level of anti-gliadin antibodies, is useful in the assessment of the presence of CD in very young children. In fact, we found that symptoms were not prognostic for CD (Table 2), indicating that the early determination of the TG2A level in genetically predisposed children may offer an opportunity for early diagnosis.[37]

The strength of our study lies in its design as a randomized, double-blind, placebo-controlled trial evaluating a food intervention in a high-risk birth cohort, with comprehensive follow-up. The cases of CD were assessed in an identical way, minimizing the risk of bias. Nonetheless, our study has some limitations. It may be argued that we introduced gluten in a rather artificial way, since 100 mg is approximately 2% of the amount normally introduced at weaning.[29] Nevertheless, this quantity has been shown previously to cause histologic damage in the intestines of CD patients.[38] After our gluten intervention, levels of anti-gliadin antibodies were transiently elevated in 59 children at 6 months of age, showing that 100 mg of gluten can indeed be immunogenic. Our power calculation was based on the assumption of a cumulative incidence of CD of 10% by 3 years of age. We found that the actual mean frequency at this age was half the assumed frequency and that it strongly depended on sex and HLA haplotype. The confidence intervals for the hazard ratio for the effect of the intervention on CD ranged from 0.79 to 1.91, indicating that we were not able to rule out a protective effect smaller than 21% or a harmful effect as large as 91%.

Our findings contrast with those from observational studies suggesting that the introduction of gluten between the ages of 4 to 6 months represents a window of opportunity for preventing CD.[23, 24] Much of the information on infant feeding and the risk of CD has been obtained from the Swedish CD epidemic, which started in the mid-1980s[21] and was related to the introduction of an increased amount of gluten after the age of 6 months, when breast-feeding became less common.[9, 22, 23, 39] However, data regarding the timing of gluten introduction in relation to breast-feeding, as well as the amount of gluten, were obtained retrospectively. Our results also contrast with recent findings in a prospective cohort of young children from the general population in Norway.[25] However, that study investigated only clinically diagnosed CD, with probable under-reporting of CD, since most cases are not clinically recognized. Whereas the observations in the Swedish and Norwegian cohorts are based on the general population from single countries, our results are derived from a study population comprising children from high-risk families in 7 European countries and Israel. Observational studies involving children with an increased risk for type

1 diabetes (positive for HLA-DQ2 or HLA-DQ8) have had controversial results. Although the results of a study conducted in the United States support the early introduction of gluten at 4 to 6 months of age,[24] the age at gluten introduction did not influence the risk of CD autoimmunity in a prospective German birth cohort.[40]

In conclusion, this randomized trial did not show the hypothesized benefit of early exposure to small quantities of gluten with regard to reducing the incidence of CD in children from high-risk families. In addition, we did not observe a reduced risk of CD associated with the maintenance of breast-feeding at the time of gluten introduction. The present European guidelines recommend the introduction of small amounts of gluten gradually while the child is breast-fed and the avoidance of both the early (<4 months) or late (>7 months) introduction of gluten.[41] Our results do not provide evidence to support these guidelines or any specific feeding recommendation with respect to the timing of gluten introduction for infants at risk for CD.

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