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Noncontaminated Dietary Oats May Hamper Normalization of the Intestinal Immune Status in Childhood Celiac Disease

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OBJECTIVES: Life-long, strict gluten-free diet (GFD) is the only treatment for celiac disease (CD). Because there is still uncertainty regarding the safety of oats for CD patients, the aim was to investigate whether dietary oats influence the immune status of their intestinal mucosa.

METHODS: Paired small intestinal biopsies, before and after >11 months on a GFD, were collected from children with CD who were enrolled in a randomized, double-blind intervention trial to either of two diets: standard GFD (GFD-std; $n = 13$) and noncontaminated oat-containing GFD (GFD-oats; $n = 15$). Expression levels of mRNAs for 22 different immune effector molecules and tight junction proteins were determined by quantitative reverse transcriptase (RT)-PCR.

RESULTS: The number of mRNAs that remained elevated was higher in the GFD-oats group ($P = 0.05$). In particular, mRNAs for the regulatory T cell (Treg) signature molecules interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1), the cytotoxicity-activating natural killer (NK) receptors KLRC2/NKG2C and KLRC3/NKG2E, and the tight junction protein claudin-4 remained elevated. Between the two groups, most significant differences were seen for claudin-4 ($P = 0.003$) and KLRC3/NKG2E ($P = 0.04$).

CONCLUSIONS: A substantial fraction of pediatric CD patients seem to not tolerate oats. In these patients, dietary oats influence the immune status of the intestinal mucosa with an mRNA profile suggesting presence of activated cytotoxic lymphocytes and Tregs and a stressed epithelium with affected tight junctions. Assessment of changes in levels of mRNA for claudin-4 and KLC3/NKG2E from onset to after a year on oats containing GFD shows promise to identify these CD patients.

INTRODUCTION

Celiac disease (CD) is an enteropathy caused by permanent intolerance to gliadin in wheat gluten and related prolamines in barley and rye.[1,2] CD affects genetically susceptible individuals carrying the major histocompatibility complex (MHC) class II alleles HLA-DQ2 and/or HLA-DQ8.[1] In CD patients, intake of dietary gluten causes an inflammatory lesion in the small intestine characterized by villous atrophy, crypt hyperplasia, and increased numbers of T lymphocytes within both the epithelium (intraepithelial lymphocytes (IELs)) and the lamina propria T lymphocytes.[3,4] Immune activity in the lesion is high, with production of both pro-inflammatory and down-regulatory cytokines by IELs and lamina propria T lymphocytes and secretion of autoantibodies of immunoglobulin A (IgA) class, e.g., anti-tissue transglutaminase (IgA-tTG) and anti-endomysium (IgA-EMA) antibodies.[4–9]

After a period of time on a gluten-free diet (GFD), there is normalization of the histology of the intestinal mucosa—the numbers of T lymphocytes decline, as does the production of cytokines and autoantibodies and the patient recovers.[10,11] There is thus far no cure for CD. Therefore, a life-long, strict adherence to a GFD is required, and this can be difficult to achieve, particularly for adolescent and pediatric celiacs.[12–14] CD patients adhering to a GFD may be at risk for inadequate intake of important dietary constituents, e.g., fiber, calcium, and vitamins.[12,15] Therefore, it would be desirable to supplement the GFD with oatmeal, both for its good nutritional profile and its contribution to a tasty diet, thereby promoting compliance to a GFD. Although the topic has been under investigation since the 1950s, it is still uncertain whether intake of oats, even when free from contamination by wheat, barley, and rye, is safe for all CD patients.[12,16] Avenin, the dominating prolamin in oat, is recognized by the immune system of CD patients as demonstrated by detection of anti-avenin antibodies in blood.[17,18] Several studies have concluded that CD patients on a GFD containing oats show

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Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

2

clinical remission, normalization of the mucosa including decline in numbers of IELs, and decreased serum titers of anti-gliadin, anti-EMA, and/or anti-tTG antibodies.[12,19] In contrast, other studies have shown that persistently elevated numbers of IELs is common among adult CD patients consuming oats,[20] and avenin-reactive T-cell lines were isolated from biopsies in five out of nine adult CD patients challenged with oats.[21] Moreover, one adult CD patient developed oat intolerance after a 1-year challenge with oat-containing GFD.[22]

Previously, we showed in a randomized, double-blind study that the majority of children with CD tolerate oat-containing GFD as determined by clinical recovery, morphologic healing of the small intestinal mucosa, and decreased autoantibody serum titers.[23] However, data on the immune status of the small intestinal mucosa after challenge with oats while on a GFD are scarce. Patients who do not gain full normalization of the local immune status on an oat-containing GFD might be at risk for recurrent disease and development of oat intolerance. In the present study our primary goal was to answer the question: Can the fact that a fraction of CD patients apparently do not tolerate oats and occasional CD patients develop overt oats intolerance be explained by disturbances in the local, intestinal immune status caused by dietary oats in these patients? As the "classical," clinical markers used for active disease do not seem sensitive enough, the second goal is to find indicators for identification of these patients at risk for oats intolerance. To achieve these goals, we here extend the analysis of the immune status of the intestinal mucosa by studying a group of patients in the previous study[23] using paired biopsies collected in active CD and after > 11 months on a GFD with or without oats. A total of 22 different mRNAs for immune effector molecules and tight junction proteins were used as indicators of ongoing mucosal inflammation.

METHODS

Ethics approval. The Human Research Ethics Committee of the Faculty of Health Sciences, Linköping University (registration number 97286, issued 1997-10-07) and the Human Research Ethics Committee of the Faculty of Medicine, Umeå University (registration number 96–304, issued 1997-01-21) approved the study. Parents of the patients gave their informed consent.

Patients and study design. This study is part of a double-blind, randomized multicenter intervention trial on the effects of including noncontaminated oats (see below) in GFD used for treatment of childhood CD. The intervention trial has been described in detail previously.[23] Patients referred and investigated because of symptoms were included consecutively and biopsied by pediatricians at 8 Swedish hospitals from June 1998 through June 2002. Random number tables were used. Each enrolled patient was given a study number, consecutively. A list for randomization of each study number to "A" or "B" diet was prepared with the allocation to "A" or "B" decided in advance. The manufacturer prepared packages and sent packages with batches of "A" and "B." The manufacturer had no other contact with any of the

participants or the patients. This guaranteed that the whole procedure was blinded for both the staff and the patients. The inclusion criteria to this part were that fresh-frozen biopsies for research had been collected both at the intervention start and at the time when the intervention was evaluated clinically, i.e., after > 11 months of GFD. All patients from whom paired biopsies were available were included. There were 15 CD patients from the group on a GFD supplemented with oats (GFD-oats) and 13 patients from the group on a standard GFD (GFD-std). In total they constituted 30% of the patients who completed the intervention. Characteristics of the patients are presented in Table 1. The serum titers of IgA-EMA and IgA-tTG were determined in blood samples drawn at the time of biopsy. All biopsies were subjected to routine histological examination of hematoxylin–eosin-stained sections for Marsh grading and IELs were counted in biopsies collected after the GFD intervention. The oats used in the study were specially grown, milled, and packaged so that they do not become contaminated with wheat, barley, or rye (Semper AB, Sundbyberg, Sweden). The median intake of oats in the GFD-oats group of this study was 20 g (range 3–43 g) at the time of control biopsy after 1 year of diet. The compliance to the dietary regimen was carefully evaluated. The parents monitored the intake of the study products, and they also kept a diary of suspected dietary mistakes. The dietician kept record of presumed consumption. Apart from the examination of the biopsy specimen after 1 year of diet, analyses of antibodies to gliadin, endomysium, and tissue transglutaminase were also performed. The clinical controls were biopsies collected on suspicion of CD from seven boys and six girls, averaging 7.2 ± 4.8 years of age (mean $\pm$ 1 s.d.), with no verified food intolerance and a normal small intestinal histology (Marsh score 0).

RNA extraction. Biopsies for gene expression analysis were snap-frozen and kept at $-70\,^{\circ}\text{C}$ until RNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Sollentuna, Sweden), according to the manufacturer's instructions, and dissolved in RNase-free water.

Real-time quantitative reverse transcriptase-PCR (qRT-PCR). Quantification of interleukin-17A (IL-17A), interferon-γ (IFN-γ), CXCL8/IL-8, IL-10, transforming growth factor-$\beta1$ (TGF-$\beta1$), tumor necrosis factor-α (TNF-α), and CX3CL1 mRNAs was performed using real-time qRT-PCR assays developed in the laboratory at Immunology, Umeå University. The assays are based on the EZ-technology, with primers placed in different exons, a reporter dye marked probe placed over the exon boundary in the amplicon, and use of an RNA copy standard. For details of the IFN-γ, IL-10, TGF-$\beta1$, and TNF-α assays, see Forsberg et al.,[6] for CXCL8/IL-8 assay, see Ou et al.,[24] and for IL-17A assay, see West et al.[25] A new assay was developed to quantify CX3CL1 mRNA. Sequences for primers and probe were 5′-CTCATC CACTATCAACAGAACCA-3′ for the forward primer, 3′-ATC GCGTCCTTGACCCA-5′ for the reverse primer, and 5′-CAA TCATCTTGGAGACGAG-3′ for the probe. Results are given as mRNA copies/μl.

Quantification of CXCL9, CXCL10, CXCL11, CXCR3, KLRB1/CD161, KLRC2/NKG2C, KLRC3/NKG2E, KLRD1/

Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

3

Table 1 Details of CD patients included in the study

Patient code	Age at inclu- sion (years)	Sex (M/F)	Duration of GFD before 2nd biopsy (months)	Serum antibodies and biopsy histology at inclusion			Serum antibodies and biopsy histology after GFD			
				IgA-EMA (titer)[a]	IgA-tTG (U/ml)[a]	PAD (Marsh score)[b]	IgA-EMA (titer)[a]	IgA-tTG (U/ml)[a]	PAD (Marsh score)[b]	IELs per 100 iECs
GFD-oats study group										
H4	4.2	F	12.2	640	73	3	0	<5	0	12
H17	0.7	M	14.3	—	29	3	0	<5	0	16
H18	11.5	F	14.5	640	>100	3	40	<5	0	24
H19	7.4	M	12.6	640	88	3	0	<5	0	14
H25	5.4	M	14.3	160	>100	3	0	<5	0	17
H41	1.2	M	13.8	—	—	3	80	11	0	ND
H46[c]	1.2	F	12.2	0[c]	<5	3	0	—	0	16
H48	1.7	F	13.4	640	>100	3	0	<5	0	14
H53	12.1	F	13.3	320	>100	3	20	<5	0	21
H54	14.2	F	12.8	80	10	3	0	<5	0	11
H59	9.5	M	12.6	640	94	3	0	<5	0	18
H60	1.7	M	14.0	1,280	>100	3	0	<5	0	14
H64	3.9	F	12.6	640	>100	3	40	9.2	0	18
H74	11.1	M	14.2	40	<5	3	0	<5	0	12
H77	2.1	F	12.6	320	>100	3	0	<5	0	19
GFD-std study group										
H1[c]	0.8	M	14.9	0[c]	<5	3	80	12	3	35
H20	6.9	F	14.0	320	64	3	0	<5	0	8
H22	1.2	M	13.8	160	65	—	0	<5	0	26
H38	3.8	F	14.4	—	—	3	0	<5	0	15
H40	6.0	M	12.2	640	>100	3	10	<5	0	22
H52[d]	8.6	F	13.0	0[d]	<5	3	0	<5	0	16
H55	12.3	F	12.8	160	75	3	0	<5	0	14
H56	1.9	F	12.6	1,280	>100	3	0	<5	0	16
H61	3.3	M	11.3	320	24	3	0	<5	0	16
H76	5.3	M	12.5	640	>100	3	0	<5	0	20
H78	2.3	F	12.8	1,280	>100	3	0	<5	0	17
H87	0.8	F	12.2	20	4.4	3	0	<5	0	20
H89	1.8	F	14.2	1,280	>100	3	20	5.7	0	19

CD, celiac disease; EMA, anti-endomysial antibodies; GFD, gluten-free diet; GFD-oats, GFD supplemented with oats; GFD-std, standard GFD; iEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A; M/F, male/female; ND, not determined; tTG, tissue transglutaminase.
The symbol "—" indicates not detectable.
[a]Cutoffs: a titer of ≥ 10 for IgA-EMA and a concentration of ≥ 5 U/ml for IgA-tTG.
[b]Biopsies were collected from June 1998 until June 2002. Final grading and counting of IEL numbers was done in a blinded manner by one experienced pathologist. An IEL count of ≥ 30 IELs per 100 IECs was considered elevated.
[c]Serum anti-gliadin IgA concentrations were determined for patients H46 and H1 and were 46 and 86 U/ml, respectively.
[d]Patient H52 had IgA deficiency. Her serum IgG-EMA titer at inclusion was 640.

CD94, inducible nitric oxide synthase (iNOS), occludin (OCLN), claudin-4 (CLDN-4), human leukocyte antigen (HLA)-A, HLA-B, HLA-C, and HLA-E mRNAs was performed using the Taqman gene Expression Assays Hs00970538_m1, Hs00171042_m1, Hs00171138_m1, Hs00171041_m1, Hs00174469_m1, Hs02379574_g1, Hs00749702_s1, Hs00233844_m1, Hs01075529_m1, Hs00170162_m1, Hs00976831_s1, Hs01058806_g1, Hs00818803_g1, Hs00740298_g1, and Hs03045171_m1, respectively (Applied Biosystems, Foster City, CA). The concentration of 18S rRNA was determined in each sample using real-time qRT-PCR (Applied Biosystems) and expressed as arbitrary units (Us) from a standard curve of serial dilutions of a preparation of total RNA from human peripheral blood mononuclear cells. One U was defined as the amount of 18S rRNA in 10 pg of RNA, and corresponds to ~100 lymphocytes.[26] The mRNA levels in RNA extracted from biopsies is 10- to 100-fold lower than in RNA extracted from purified lymphocytes as a consequence of the multitude of different cell types present in the biopsy tissue. All samples included in the study contained >16 Us 18S rRNA per reaction mixture.

All quantitative determinations were performed in triplicate using the ABI prism 7700 sequence detection system (Applied Biosystems). The mRNA concentrations were normalized to the 18S rRNA concentration in the sample by calculating mRNA copies/18S rRNA U for mRNAs analyzed by assays with an RNA copy standard and by calculating the ΔCT between the CT for the mRNA species and the CT for 18S rRNA (CT_{sample}–$CT_{18S\ rRNA}$) for mRNAs analyzed by commercial Taqman gene Expression Assays. The results for genes analyzed by Taqman gene Expression Assays are given as relative quantity calculated as $2^{(-\Delta\Delta CT)}$ where $\Delta\Delta$CT is ΔCT for the sample minus the median of the ΔCT values of the samples from control patients ($n = 13$).

Statistical analysis. Statistical analyses were performed using the Prism 5 computer program (GraphPad Software, San Diego, CA). Statistical analysis of differences in mRNA levels in paired samples before and after GFD was

Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

4

performed using a paired, nonparametric Wilcoxon t-test. Differences between CD patient groups and between CD patient groups and controls were performed using a nonparametric Mann–Whitney U-test. Comparisons of frequencies in groups were performed using Fisher's exact test. Results of parameters with Gaussian distribution as estimated by Kolmogorov–Smirnov normality test are given as mean ± 1 s.d. and otherwise as median and interquartile range. Two-sided analysis was used throughout. A P value of ≤ 0.05 was regarded as statistically significant.

RESULTS

Characteristics of the study groups. A total of 28 children with newly diagnosed, symptomatic CD were enrolled and randomized into a double-blinded study comparing treatment with a GFD with noncontaminated oats (GFD-oats, $n = 15$; 7 boys and 8 girls; age 5.8 ± 4.7 years) and a standard GFD without oats (GFD-std, $n = 13$; 5 boys and 8 girls; age 4.2 ± 3.5 years, $P > 0.05$). Small intestinal biopsies were collected from each child within 4 weeks before the study diet was introduced and after > 11 months on a GFD with and without oats (range: 11.3–14.9 months; mean ± 1 s.d.: 13.3 ± 0.8 and 13.1 ± 1.0 months in the GFD-oats and the GFD-std group, respectively, $P > 0.05$, Table 1). The diagnosis of all enrolled CD patients except one was verified by biopsy histology (Marsh score 3 at first biopsy) and with three exceptions in each study group supported by an IgA-EMA titer above 10 (Table 1). There was no significant difference in IgA-EMA and IgA-tTG titers, and small intestinal histology score between the two study groups before the GFD intervention (IgA-EMA titer 431 ± 365 and 555 ± 511, IgA-tTG 66 ± 44 and 61 ± 42 U/ml and 15 of 15 and 12 of 13 with Marsh score 3 in the GDF-oats and GFD-std groups, respectively, $P > 0.05$). None of these parameters or the IEL counts differed significantly between the two study groups after the intervention (16.1 ± 3.7 and 18.8 ± 6.5 IELs per 100 epithelial cells for the GFD-oats and GFD-std groups, respectively, $P > 0.05$).

Analysis of changes in the expression levels of mRNAs for immune effector molecules and tight junction proteins in small intestinal biopsies from patients treated with GFD-oats and GFD-std, respectively, before and after the intervention. Before intervention, there was no significant difference between the mRNA levels for any of 22 different immune effector molecules and tight junction proteins in the two study groups, i.e., GFD-oats and GFD-std (Supplementary Table S1 and Supplementary Figure S1 online). After the intervention, however, the two study groups differed for several mRNAs with regard to how individuals had responded. In the following text, we report the results according to functional molecular classes, i.e., proinflammatory and downregulatory cytokines, chemokines, natural killer (NK) receptors, and MHC class I molecules, and tight junction proteins.
Proinflammatory and downregulatory cytokines. IL-17A, IFN-γ, IL-10, and TGF-β1 transcripts levels were used as estimates of Tc17, Th17, Tc1, Th1, and Treg activities. Figure 1 and Supplementary Figure S1 show mRNA values

for individual patients of the two study groups before and after intervention. Statistical comparisons were performed by comparing individual changes in the two study groups using a two-sided Wilcoxon matched pair t-test and statistically significant values are shown in Figure 1. Only individuals who had a detectable mRNA value of a particular cytokine before intervention were included in the comparisons. The two groups did not differ in number of individuals with detectable mRNA before intervention ($P > 0.05$). IL-17A mRNA levels correlated with disease activity in each individual in both diet groups with significantly reduced levels close to those of controls after GFD (Figure 1a and Supplementary Table S2). Analysis of IFN-γ mRNA levels gave a similar picture. However, although IFN-γ levels were reduced after GFD in both diet groups, the reduction did not reach statistical significance in the GFD-std group ($P = 0.07$; Figure 1b and Supplementary Figure S1). Before GFD intervention, IL-10 transcript levels were significantly higher in CD patients than in controls in both study groups ($P < 0.01$; compare Supplementary Tables S1 and S2), but showed significant reduction only after GFD-std (Figure 1c). Several patients even had higher IL-10 mRNA levels on GFD-oats, compared with the intervention start (Figure 1c and Supplementary Figure S1). TGF-β1 mRNA levels showed a similar picture to that of IL-10, with significant reduction only after GFD-std (Figure 1d and Supplementary Figure S1).

Levels of mRNAs for the proinflammatory cytokine TNF-α and the inflammatory and antimicrobial enzyme iNOS were used as indicators of activated macrophages and/or epithelial cells.[27,28] Average TNF-α mRNA levels before intervention were only marginally increased compared with controls (compare Supplementary Tables S1 and S2). Nevertheless, all patients in the GFD-std group with detectable TNF-α mRNA in the first biopsy showed a significantly lower TNF-α mRNA level after GFD, whereas several patients in the GFD-oats group showed an increased level (Figure 1e and Supplementary Figure S1). The mRNA for iNOS was on average threefold higher than in controls in both groups (compare Supplementary Tables S1 and S2). The iNOS transcripts were significantly reduced after GFD in both groups, but the reduction was more pronounced in the GFD-std group, with a decline in all but one patient (Figure 1f). Thus, mRNA for three of five cytokines, IL-10, TGF-β1, and TNF-α, did not normalize in several patients on GFD with oats. *Chemokines.* The chemokines CX3CL1, CXCL8/IL-8, CXCL9, CXCL10, and CXCL11 have all been implicated in inflammatory bowel disease and are involved in recruitment of immune cells to sites of inflammation and infection.[29–32] We analyzed these five chemokines and CXCR3, a receptor for CXCL9–11. The average levels of all five chemokines were significantly higher in CD patient biopsies before GFD compared with controls ($P < 0.01$; compare Supplementary Tables S1 and S2). After GFD, all CD patients except for one or two in each diet group showed a drastic decline in chemokine mRNAs that reached the levels of controls (Figures 2a–e). CXCR3 was expressed at low levels and was not detected in all CD patients (Supplementary Table 1 and Supplementary Figure S1). CXCR3 mRNA levels in active CD were on average lower than in controls and even though levels tended to increase after GFD they were still
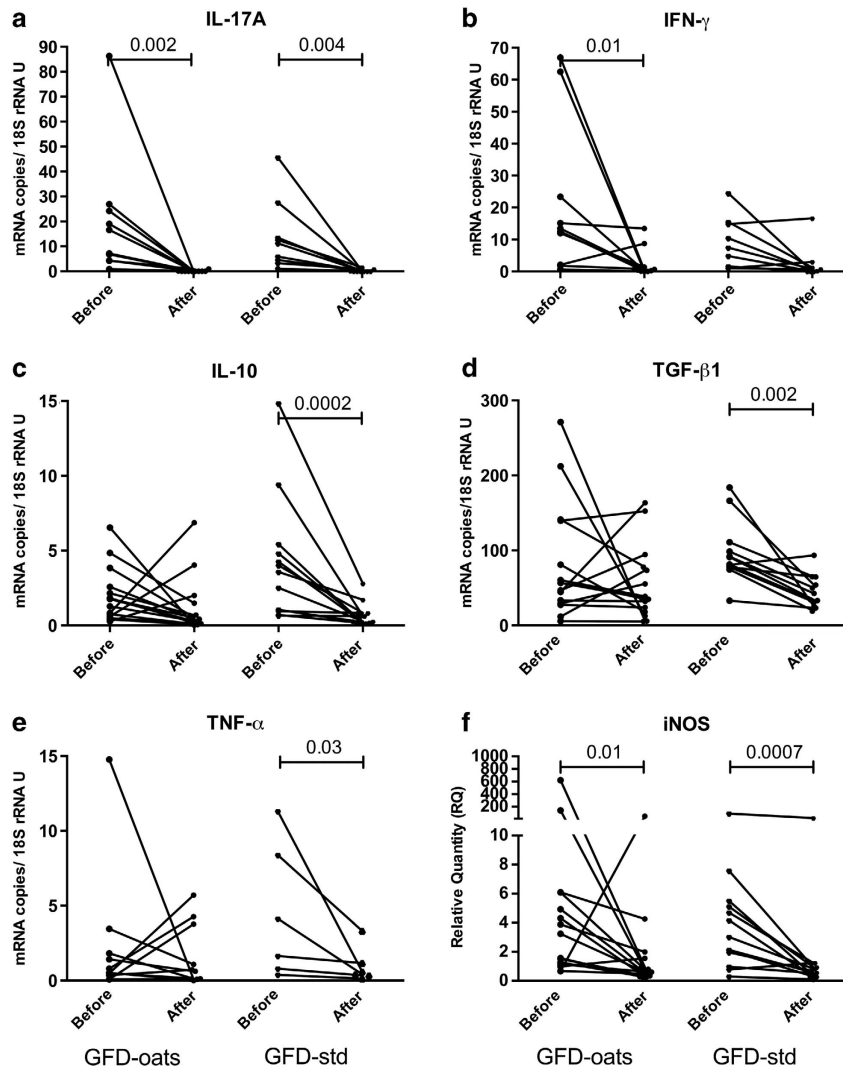
Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

5

Figure 1 Cytokines and an inflammatory effector molecule. Expression levels of mRNAs for (**a**) interleukin (IL)-17A ($n = 10$ and 9 for the GFD-oats and GFD-std groups, respectively), (**b**) interferon-γ (IFN-γ; $n = 10$ and 9), (**c**) IL-10 ($n = 15$ and 13), (**d**) transforming growth factor-β1 (TGF-β1; $n = 15$ and 12), (**e**) tumor necrosis factor-α (TNF-α; $n = 11$ and 6), and (**f**) inducible nitric oxide synthase (iNOS; $n = 15$ and 13) in small intestinal biopsies of celiac disease (CD) patients before (Before) and after (After) gluten-free diet (GFD) with oats (GFD-oats) or standard GFD without oats (GFD-std). Amounts of IL-17A, IFN-γ, IL-10, TGF-β1, and TNF-α mRNAs were determined by quantitative reverse transcriptase-PCR (qRT-PCR) with an RNA copy standard and normalized to the 18S rRNA content in the sample (mRNA copies/18S rRNA arbitrary unit (U)). Amounts of iNOS mRNA were determined by qRT-PCR with no copy standard and normalized to 18S rRNA in the sample by calculating the ΔCT value between the mRNA and the 18S rRNA. The relative quantity (RQ) of iNOS mRNA was calculated as $2^{(-\Delta\Delta CT)}$ where $\Delta\Delta$CT is the ΔCT value for the sample minus the median ΔCT-value in biopsies of 13 clinical controls. Each point represents a single patient before and after GFD. Lines connect the mRNA levels in biopsies of single CD patients before and after GFD. Statistically significant differences in mRNA levels before and after GFD are indicated. Statistical comparisons were performed by using two-sided Wilcoxon matched paired t-test.

below those of controls (compare Supplementary Tables S1 and S2). Thus, mRNA levels for all five chemokines, CX3CL1, CXCL8/IL-8, CXCL9, CXCL10, and CXCL11, followed disease activity with no difference between the two GFD diets.

NK receptors and MHC class I molecules. Active CD is associated with increased cytotoxicity of IELs and increased frequencies of KLRD1/CD94-expressing IELs.[33,34] We therefore analyzed the expression levels of mRNAs for KLRD1/CD94, KLRC2/NKG2C, and KLRC3/NKG2E. The latter two molecules are alternative subunits that together with KLRD1/CD94 form the activating NK receptors that use

HLA-E as ligand.[35,36] Levels of HLA-E mRNA and the polymorphic MHC class I molecules HLA-A, -B, and -C mRNAs were analyzed for comparison. Finally, the NK receptor KLRB1/CD161 was also analyzed as it has been suggested to play a role in CD.

In controls, levels of KLRC2/NKG2C mRNA were generally low and only detected in 6 of the 13 individuals (Supplementary Table S2), whereas in CD patients before GFD, it was significantly higher and detected in the majority of biopsies (Supplementary Figure S1 and compare Supplementary Tables S1 and S2). All patients in the GFD-std group showed a significant reduction of KLRC2/NKG2C
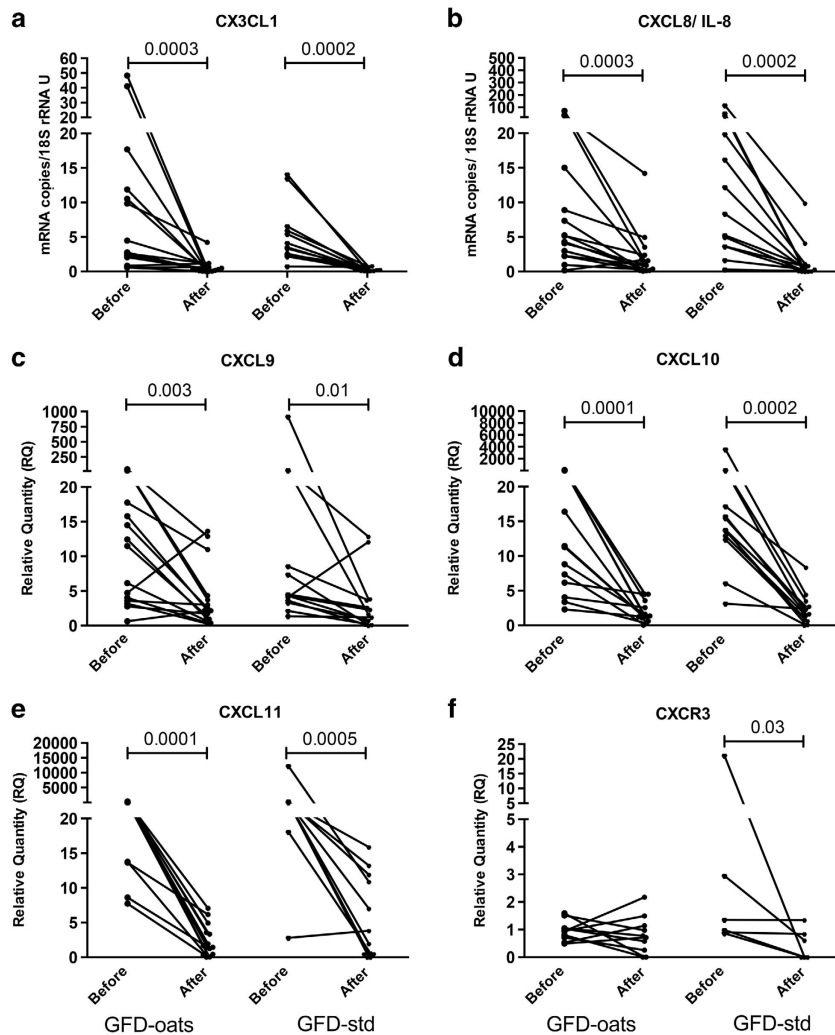
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Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

6

Figure 2 Chemokines and a chemokine receptor. Expression levels of mRNAs for (**a**) CX3CL1 ($n = 15$ and 13 for the gluten-free diet (GFD)-oats and GFD-std groups, respectively), (**b**) CXCL8/interleukin-8 (IL-8) ($n = 15$ and 13), (**c**) CXCL9 ($n = 15$ and 13), (**d**) CXCL10 ($n = 14$ and 13), (**e**) CXCL11 ($n = 14$ and 13), and (**f**) CXCR3 ($n = 10$ and 6) in small intestinal biopsies of celiac disease (CD) patients before (Before) and after (After) a GFD with oats (GFD-oats) or standard GFD without oats (GFD-std). Amounts of CX3CL1 and CXCL8/IL-8 mRNAs are given as mRNA copies/18S rRNA arbitrary unit (U). Amounts of CXCL9, CXCL10, CXCL11, and CXCR3 are given as relative quantity (RQ) compared with the median ΔCT-value of 13 clinical controls. For further details, see legend for Figure 1.

mRNA after GFD treatment, whereas this was not the case in the GFD-oats group (Figure 3a). KLRC3/NKG2E mRNA was readily detected in all biopsies and expressed at significantly higher levels in patients with active CD than in controls (Supplementary Figure S1 and compare Supplementary Tables S1 and S2). A marked reduction of KLRC3/NKG2E mRNA was seen in all but one patient on GFD-std but only in 8 of the 15 patients in the GFD-oats group (Figure 3b). Interestingly, after the GFD period, CD patients in both study groups still had significantly higher levels of KLRC3/NKG2E mRNA than controls (Supplementary Table S2). KLRD1/CD94 mRNA was detected in all biopsies although the level was low and not higher in active CD compared with controls (Figure 3c). Although KLRD1/CD94 mRNAs before GFD were not significantly increased in active CD ($P > 0.05$), there was a marginal decline after GFD in the GFD-oats group (Figure 3c). In spite of the fact that HLA-E mRNA was generally expressed

at lower levels in CD patients than in controls (Supplementary Tables S1 and S2), there was a significant reduction in HLA-E mRNA levels after GFD in both groups (Figure 3d). In contrast, HLA-A, -B, and -C mRNAs were expressed at high levels and did not change with disease activity in either group (Supplementary Figures S1 and S2a–c). KLRB1/CD161 mRNA was detected in all biopsies but on average was lower than in controls (Supplementary Figure S1 and Supplementary Tables S1 and S2) and the levels did not change after GFD in either group (Supplementary Figure S2d). Thus, normalization of KLRC2/NKG2C and KLRC3/NKG2E mRNA levels did not occur to the same extent on the GFD-oats diet as on the GFD-std diet. In fact, some individuals even showed increased levels.

Tight junction proteins. Increased epithelial permeability has been proposed in CD.[37] CLDN-4 and OCLN are the main components of the epithelial tight junctions[38] and were

Oats Affect Local Immunity in Some Celiac Disease Patients
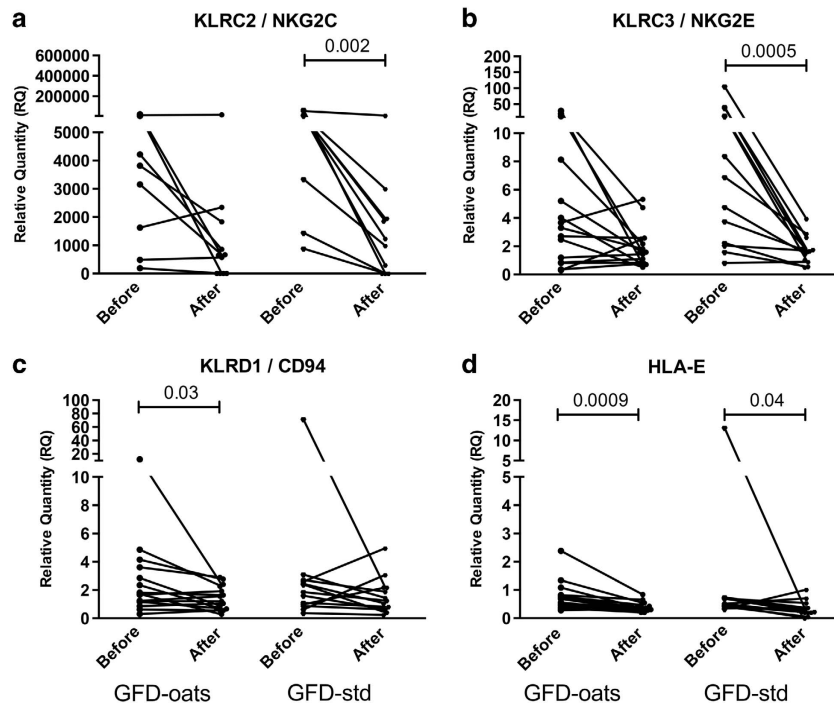Sjöberg et al.

7

Figure 3 Natural killer (NK) receptors and a NK receptor ligand. Expression levels of mRNAs for (**a**) KLRC2/NKG2C ($n = 11$ and 10 for the GFD-oats and GFD-std groups, respectively), (**b**) KLRC3/NKG2E ($n = 15$ and 13), (**c**) KLRD1/CD94 ($n = 15$ and 13), and (**d**) HLA-E ($n = 15$ and 13) in small intestinal biopsies of celiac disease (CD) patients before (Before) and after (After) a gluten-free diet (GFD) with oats (GFD-oats) or standard GFD without oats (GFD-std). mRNA levels are given as relative quantity (RQ) compared with the median ΔCT-value of 13 clinical controls. For further details, see legend for Figure 1.

therefore analyzed. Average CLDN-4 mRNA levels were higher in active CD than in controls (Supplementary Tables S1 and S2) and showed significant decline after GFD-std but not after GFD-oats (Figure 4a). OCLN levels in CD patients were not significantly different from those of controls either before or after GFD in either study group (Figure 4b and Supplementary Tables S1 and S2).

Markers for incomplete normalization. The immune status in the mucosa of the patients after intervention was assessed as the number of mRNAs with levels that had decreased compared with intervention start. In the analysis, we only included those mRNA species ($n = 17$) that showed changes related to disease activity in either or both diet groups. A median of 2 (interquartile range: 1–4.5) mRNAs were unchanged or increased in the GFD-std group as compared with a median of 5 (interquartile range: 3–6) in the GFD-oats group ($P = 0.05$; Figure 5). There was no trend for increased anti-EMA titer, anti-tTG titer, IEL count, or Marsh score in patients who had high numbers of mRNAs with unchanged/increased levels (compare Table 1). Using the median for the number of mRNA species that had not decreased after intervention in the GFD-std group, i.e., < 2 mRNA species with unchanged or increased levels, as cutoff for a normalized mucosa, it was found that the number of patients with normalized mucosa was significantly lower in the GFD-oats group, i.e., 1 of 15 compared with 6 of 13 patients in the GFD-oats and GFD-std groups, respectively (Figure 5; $P = 0.03$). It should be noted, however, that the

average level for individual mRNA species did not differ significantly between the clinical controls and the GFD-oats group after intervention, mainly because the GFD-oats group was heterogeneous and some individuals responded with decreased mRNA levels to the GFD whereas others did not (Supplementary Table S2).

The mRNA species that showed the most pronounced difference with respect to decreased levels after GFD between the two study groups were CLDN-4 and KLRC3/NKG2E. In the GFD-std group, CLDN-4 mRNA levels decreased in all patients and KLRC3/NKG2E mRNA levels decreased in all but one patient. In contrast, in the GFD-oats group, seven patients remained high for both mRNAs and one patient remained high for CLDN-4 only (Figure 5, $P = 0.04$ and 0.003 for KLRC3/NKG2E and CLDN-4, respectively).

DISCUSSION

The major outcome of this study is that the immune status in the small intestinal mucosa is not normalized in a substantial fraction of pediatric CD patients on a GFD containing oats. That the failure to normalize the immune status was indeed a consequence of intake of oats was underscored by the fact that the dietary oats utilized were free from contamination by wheat, barley, and rye. However, only certain aspects of mucosal immunity seem to be affected. Thus, although the mRNA expression levels of the proinflammatory cytokines IL-17A and IFN-γ and the chemokines CX3CL1, CXCL8/IL-8,
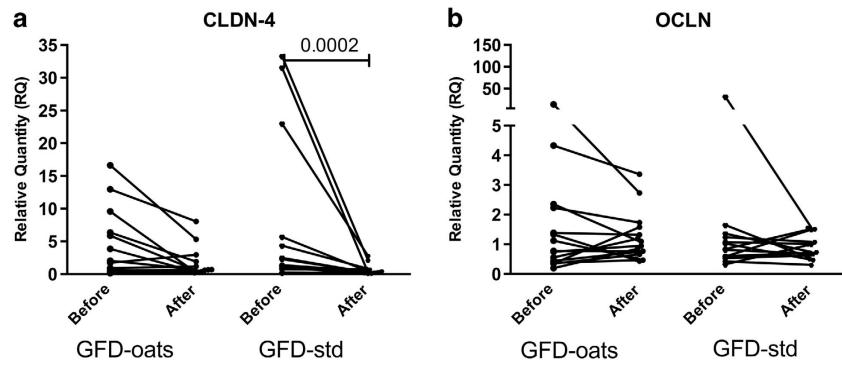
Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

8

Figure 4 Epithelial tight junction components. Expression levels of mRNAs for (**a**) claudin-4 (CLDN-4; $n = 15$ and 13 for the GFD-oats and GFD-std groups, respectively) and (**b**) occludin (OCLN; $n = 15$ and 13) in small intestinal biopsies of celiac disease (CD) patients before (Before) and after (After) a gluten-free diet (GFD) with oats (GFD-oats) or standard GFD without oats (GFD-std). Amounts of mRNAs are given as relative quantity (RQ) compared with the median ΔCT-value of 13 clinical controls. For further details, see legend for Figure 1.

Figure 5 Influence of gluten-free diet (GFD) on the cytokine profile of each individual celiac disease (CD) patient, indicated by their patient code (H-number), in the two study-groups receiving either GFD with oats (GFD-oats) or GFD without oats (GFD-std). The 17 mRNA species that changed after the GFD intervention are shown. An arrow pointing down in a green box indicates a decreased mRNA level after intervention with GFD, i.e., the expression level in the second biopsy < 90% of the expression level in the first biopsy. An arrow pointing upward in a red box indicates an unchanged or increased mRNA level after intervention with GFD, i.e., the expression level in the second biopsy ≥ 90% of that in the first biopsy. UD (undetected) written in a yellow box indicates that the mRNA species was not detected in either one of the two biopsies, i.e., neither before nor after GFD. Normalized: < 2 mRNA species unchanged or increased after GFD.

CXCL9, CXCL10, and CXCL11 were fully normalized in these patients, this was not the case for markers of downregulatory and cytotoxic activities. First, levels of the downregulatory cytokine IL-10—previously shown to follow disease activity, particularly in CD8^{+} IELs in pediatric CD patients[6,9,39]—did not normalize in the GFD-oats group. The same result was seen with TGF-β1, the second down-regulatory cytokine investigated. These results are indicative of ongoing immune activities in the intestinal mucosa of patients in the GFD-oats group, i.e., that Tregs are actively

trying to downregulate certain immune activities. Second, the two activating NK receptors KLRC2/NKG2C and KLRC3/ NKG2E were both upregulated in active CD. Only the GFD-std group showed a statistically significant decrease in the levels of these mRNAs on GFD. In contrast, half the number of patients in the GFD-oats group had persistently elevated levels of KLRC3 mRNA. This indicates that cytotoxic T lymphocytes are still active in the mucosa in patients on GFD-oats. Previous studies have shown that the epithelium is the main site for cytotoxic lymphocytes in the human small intestinal mucosa in both healthy individuals and CD patients,[34,40,41] and that the proportion of IELs expressing KLRD1/CD94 is increased in active CD.[33] Taken together, our data suggest that highly cytotoxic IELs persist in a considerable fraction of CD patients consuming oats. We interpret these results as an indication that the epithelium is still stressed, although more subtly than in active disease. Indeed, the third pronounced difference between the two patient groups was that mRNA for the tight junction protein CLDN-4 did not normalize in the GFD-oats group. Thus, it appears that oats have adverse effect on the epithelium in CD patients. Active CD is suggested to be associated with increased epithelial permeability and cross-epithelial transport of gliadin peptides.[42] It could be speculated that certain individuals have a more pronounced tendency to develop a leaky epithelium. If so, presumably these individuals are not able to stand the milder epithelium stress induced by oats. TNF-α and iNOS mRNA levels had not normalized in some patients in the GFD-oats group, supporting the notion of a stressed epithelium and failure to fully recover from the inflammatory reaction. Persistent iNOS activity in patients in the GFD-oats group was also indicated when measuring NO metabolites in the urine of the same study population.[43] CLDN-4 and KLRC3/NKG2E seem to be the best discriminators between those whose immune status normalizes on an oat-containing GFD and those whose immune status does not.

Collectively, our results support the notion that a substantial fraction of CD patients tolerate oats poorly. In contrast, several studies, including the randomized double-blind study of which the patients in the present study constitute a subsample, have shown that there is no significant difference between CD patients who have been on GFD with or without oats with respect to frequencies of patients who recovered symptomatically, and had normalized mucosal architecture and normalized serum titers of anti-gliadin, anti-EMA, and anti-tTG antibodies.[12,23,44] There are, however, discrepancies in the literature with regard to effects of oat-containing diet on IEL counts, reporting either no influence or significantly higher IEL counts in CD patients on oats-containing diet.[16] Recently, Ilus *et al.*[20] reported that high IEL counts in patients who had been on a long-term, strict GFD could only be explained by consumption of oats. These observations are in line with results from the present study as they point toward altered epithelial function and presence of activated IELs in $\sim50\%$ of CD patients on oat-containing GFD. Moreover, Lundin *et al.*[21,22] reported that oats-containing GFD induced villous atrophy and avenin-reactive, HLA-DQ2-restricted intestinal T cells in a subgroup of adult CD patients and oat intolerance

in one of them. Some gliadin-reactive T lymphocytes crossreact with secalin and hordein, the prolamines in rye and barley.[45] Thus, it would appear as if even avenin can cause an immune response in the intestinal mucosa in some individuals, either on its own or by crossreactivity. The reason why only a fraction of CD patients respond adversely to oats is not known, but in view of previous reports on increased IEL counts and the results from the present study that suggest altered functions of the epithelium, it is conceivable that sensitivity of the epithelial lining to oats components is one determining factor. This could occur by direct action of oats on the epithelial cells or oats could contribute to dysbiosis in the resident microbiota of the small intestine. Regardless, it appears that IELs stay in a state of high cytotoxic potential in this subgroup of CD patients. The long-time effect of this cannot be evaluated in this study. Hypothetically, these patients might be at risk for complications associated with CD in adulthood, such as refractory sprue and malignancy.

The limitations of the present study are that paired fresh-frozen samples were available only from a limited number of patients, that the follow-up time of 1 year does not allow evaluation of consequences of long-time exposure to dietary oats, and finally that all study subjects were children and hence we do not know as yet whether oats can have adverse effects on the intestinal immune status in adult CD patients also. Thus, the evaluation of the long-time effect of the high cytotoxic potential of IELs caused by noncontaminated dietary oats lies beyond the scope of this study, but substantiates the need for future follow-up. In a review on the safety of including oats in GFD for CD patients, Haboubi *et al.*[16] concluded that ''Patients with coeliac disease wishing to consume a diet containing oats should therefore receive regular follow-up, including small bowel biopsy at a specialist clinic for life.'' Although the present study is small and needs to be repeated using clinical material from a larger cohort of patients, our results support their conclusion. Diagnostic procedures must be adjusted to practice in different countries but a possible scenario based on our results is to suggest that all patients who want to try a GFD containing oats should be subjected to a second biopsy after >1 year, or earlier if gastrointestinal complaints occur. We propose that it should be possible to discriminate between pediatric CD patients who tolerate oats and those who do not by determining the levels of mRNAs— in particular, CLDN-4, KLRC3/NKG2E, and IL-10—that do not decrease in the control biopsy after 1 year on GFD containing oats.

CONFLICT OF INTEREST

Oats Affect Local Immunity in Some Celiac Disease Patients
Sjöberg et al.

10

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✔ In celiac disease (CD) patients, dietary gluten causes a small intestinal lesion.
- ✔ The lesion resolves and the immune status normalizes with a gluten-free diet (GFD).
- ✔ Supplementing a GFD with oats is desirable for its nutritional value and taste.
- ✔ Case reports of CD patients on a GFD containing oats who have developed oat intolerance.

WHAT IS NEW HERE

- ✔ Intestinal immune status does not normalize on oats-containing GFD in a substantial fraction of CD patients.
- ✔ KLRC3/NKG2E and claudin-4 are good mucosal markers of CD patients sensitive to dietary oats.

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Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)