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Bentz, S ; Hausmann, M ; Piberger, H ; Kellermeier, S ; Paul, S ; Held, L ; Falk, W ; Obermeier, F ;  
Fried, M ; Schölmerich, J ; Rogler, G

**Abstract:** Background: Environmental factors are thought to play an important role in the development of Crohn's disease (CD). Immune responses against auto-antigens or food antigens may be a reason for the perpetuation of inflammation. Methods: In a pilot study, 79 CD patients and 20 healthy controls were examined for food immunoglobulin G (IgG). Thereafter, the clinical relevance of these food IgG antibodies was assessed in a double-blind cross-over study with 40 patients. Based on the IgG antibodies, a nutritional intervention was planned. The interferon (IFN) secretion of T cells was measured. Eosinophil-derived neurotoxin was quantified in stool. Results: The pilot study resulted in a significant difference of IgG antibodies in serum between CD patients and healthy controls. In 84 and 83% of the patients, respectively, IgG antibodies against processed cheese and yeast were detected. The daily stool frequency significantly decreased by 11% during a specific diet compared with a sham diet. Abdominal pain reduced and general well-being improved. IFN secretion of T cells increased. No difference for eosinophil-derived neurotoxin in stool was detected. Conclusion: A nutritional intervention based on circulating IgG antibodies against food antigens showed effects with respect to stool frequency. The mechanisms by which IgG antibodies might contribute to disease activity remain to be elucidated.

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# Clinical Relevance of IgG Antibodies against Food Antigens in Crohn's Disease: A Double-Blind Cross-Over Diet Intervention Study

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## Key Words

Crohn's disease · Immunoglobulin G antibodies · Food antigen · Interferon

## Abstract

**Background:** Environmental factors are thought to play an important role in the development of Crohn's disease (CD). Immune responses against auto-antigens or food antigens may be a reason for the perpetuation of inflammation. **Methods:** In a pilot study, 79 CD patients and 20 healthy controls were examined for food immunoglobulin G (IgG). Thereafter, the clinical relevance of these food IgG antibodies was assessed in a double-blind cross-over study with 40 patients. Based on the IgG antibodies, a nutritional intervention was planned. The interferon (IFN) $\gamma$  secretion of T cells was measured. Eosinophil-derived neurotoxin was quantified in stool. **Results:** The pilot study resulted in a significant difference of IgG antibodies in serum between CD patients and healthy controls. In 84 and 83% of the patients, respectively, IgG antibodies against processed cheese and yeast were detected. The daily stool frequency significantly decreased by 11% during a specific diet compared with a sham diet. Abdominal pain reduced and general well-being improved. IFN $\gamma$  secretion of T cells increased. No difference for eosinophil-derived neurotoxin in stool was detected. **Con-**

**clusion:** A nutritional intervention based on circulating IgG antibodies against food antigens showed effects with respect to stool frequency. The mechanisms by which IgG antibodies might contribute to disease activity remain to be elucidated.

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## Introduction

Genetic influences [1, 2], cytokine activation [3] and various specific and nonspecific environmental factors like hygiene, social standard, climatic factors, environmental pollution, smoking, stress and nutrition [4–7] have been considered to be associated with the induction and/or exacerbation of inflammatory bowel disease (IBD), such as Crohn's disease (CD).

Serologic markers for IBD, like anti-*Saccharomyces cerevisiae* antibodies and atypical perinuclear antineutrophil cytoplasmic antibodies, remain to play a role in the pathophysiology of IBD. There is a wide range of other antibodies including outer-membrane porin C, anti-*Pseudomonas fluorescens* and antiglycan antibodies (anti-laminaribioside carbohydrate antibody, anti-chitobioside carbohydrate antibody, anti-mannobioside carbohydrate antibody), and anti-CBir1. The latter is the

first bacterial antigen found to induce colitis in animal models of IBD and also leads to a pathological immune response in IBD patients [8].

Frequently, IBD patients report that dietary intolerance significantly contributes to their symptomatology. The benefit from eliminating certain foods [9] from daily diet was refocused in the present study. Attempts to test for food intolerance in IBD have largely focused on classic food allergies based on the presence of immunoglobulin E (IgE)-mediated antibody responses, although these reactions appear probably quite rare in IBD [10]. It is therefore possible that adverse reactions in IBD might be due to some reactions mediated by IgG antibodies, which characteristically give a more delayed response following exposure to a particular antigen [11] and have been implicated in some cases of food hypersensitivity [12–14]. However, this mechanism is controversial and is considered to be physiological [15–17], as IgG food antibodies can be present in apparently healthy individuals [18, 19]. It has been assumed that chronic inflammation in IBD is due to an imbalance between inflammatory and anti-inflammatory mechanisms like regulatory CD4+CD25+ T cells (T<sub>regs</sub>) [20–22].

High IgG levels against certain food components in blood and the inflammatory response of T cells to food antigens and the regulatory effect of T<sub>regs</sub> in vitro was assessed. As IgG food antibodies may play a role during the initiation or perpetuation of IBD, we first investigated the presence of IgG antibodies in CD patients and healthy controls. In a second approach, the therapeutic potential of an elimination diet based on the presence of IgG antibodies to food in patients with CD in a randomized controlled trial was investigated. Primary outcome parameters were stool frequency, abdominal pain and general well-being. The possible activation of T-effector cells through IgG antibodies was measured by interferon (IFN) $\gamma$  secretion. For the evaluation of disease activity, eosinophil-derived neurotoxin (EDN) was quantified in stool. A secondary outcome parameter was the total score built from stool frequency, abdominal pain and general well-being.

## Methods

### *Study Design 1: Pilot Study*

Initially, 20 healthy volunteers without history of food intolerance and 79 CD patients with different disease status were included in a pilot study. Forty-seven of them had clinical and endoscopic signs of acute inflammation (i.e. diarrhea and mucosal ulcerations). Twenty-four CD patients presented with chronically

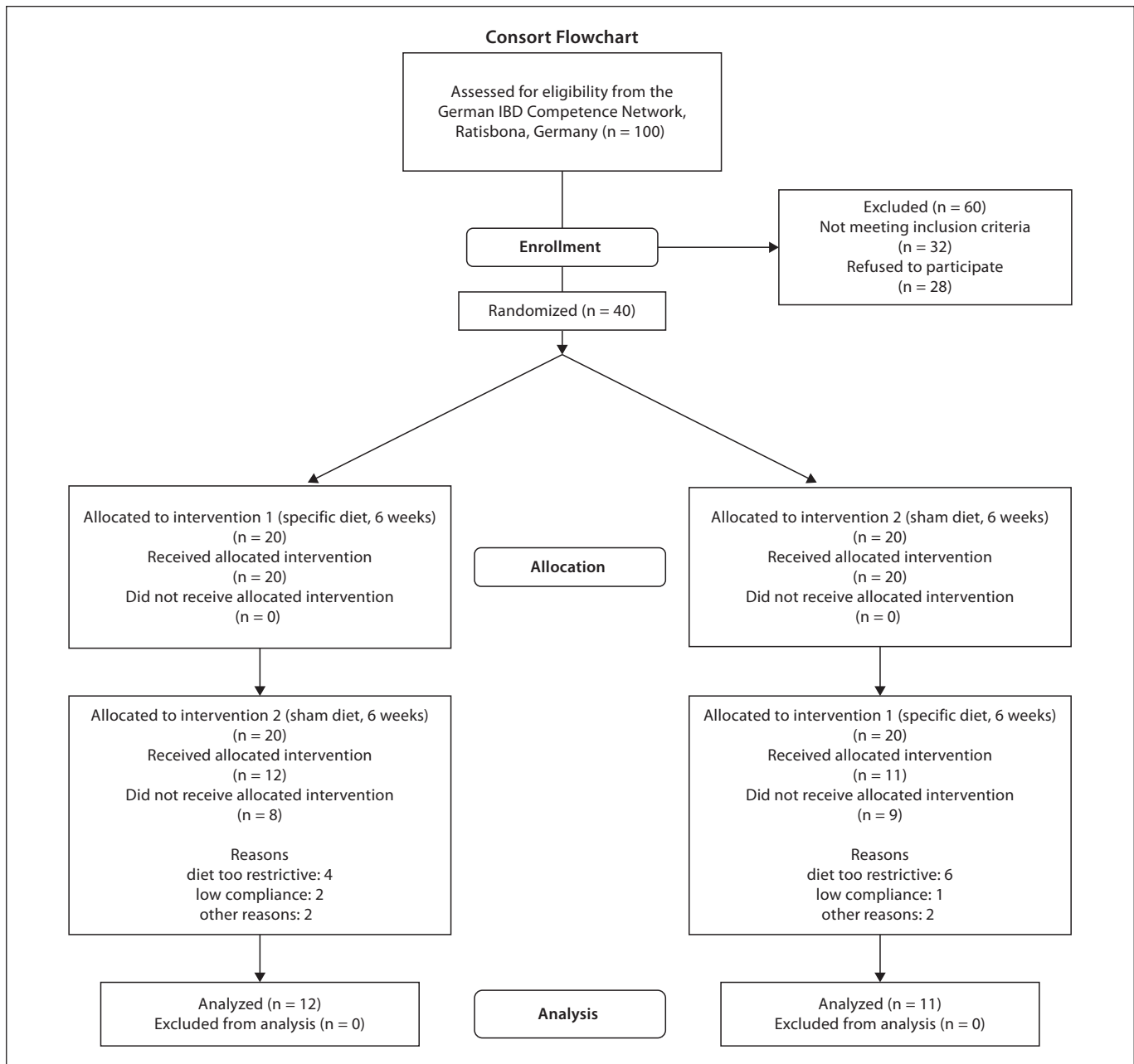
active disease and 8 were in remission. Patients were recruited from the German IBD Competence Network serum bank and examined for food specific IgG by the ImuPro300 test (Evomed, Darmstadt, Germany). Disease activity was assessed by the patient's medical record.

### *Study Design 2: Following Intervention Study*

Consecutively in a randomized, double-blind, cross-over intervention study, the clinical relevance of IgG antibodies against food antigens in 40 CD patients was tested. Not all patients from the previous pilot study were willing to participate in the following intervention study; therefore, new patients were also tested for food IgG antibodies in serum. Patients were not selected for IgG levels in serum. A sample size calculation was not performed. Finally, the specific antibody pattern of 40 patients was determined in serum samples by the ImuPro300 test system. The reactivity of T<sub>regs</sub> and CD4+CD25- T cells to the patient-specific food antigens was determined in vitro (mixed lymphocyte stimulation assay) and correlated with in vivo changes on the basis of a nutritional record and a patient diary. The diary contained questions about stool frequency, abdominal pain and general well-being. Patients validated their pain perception with scores of 0, 1, 2 and 3 which represented no pain, slight pain, moderate pain and severe pain, respectively. The values were accumulated after each week. Additionally, the patients rated their general well-being. The patients assessed general well-being by a score of 0, 1, 2, 3 and 4, which represented good, worse, bad, very bad and terrible, respectively. To get an overall impression of the symptoms, stool frequency, abdominal pain and general well-being, a total score was calculated. Each subject recorded his eating habits and disease symptoms over a period of 12 weeks and followed a specific or sham diet. Each diet was followed for 6 weeks (fig. 1). The definition of specific and sham diet was based on similarity of excluded food components. If, for example, IgG against hazelnut was detected, then almond was excluded in the sham diet; if cauliflower IgG was found, broccoli was excluded. Patients were concealed and allocated to one of the two diet sheets based on a randomization schedule using a random computer number generator. Thus, patients received either an elimination diet based on their true sensitivity results (specific diet) or a sham diet. Baseline demographic and clinical characteristics of the two groups, including the use of concomitant medication, were found to be similar. All patients and clinical staff in the gastroenterology research department were blinded to the group assignment of all patients for the duration of the study. Patients were given their allocated diet sheet by staff at the gastroenterology department and asked to eliminate the indicated foods from their diet for a period of 12 weeks. They also received a booklet with advice on how to eliminate the different foods (recipes and menus). This was explained by an experienced nutritionist. Furthermore, the telephone contact details of a free nutritional advisor who they could contact for further advice if necessary was given to each patient.

### *Subjects*

There were 16 male and 24 female subjects. Ultimately, data analysis of 23 patients was performed. Patients between the ages of 18 and 60 years were considered eligible. In this study, the patients were between 21 and 59 (mean 41  $\pm$  11) years of age. Both active and inactive patients were included, and diagnosis was manifested at least 6 months before onset of the trial. Duration of



**Fig. 1.** Study flow of the intervention trial. Patients were allocated to one of the two diets: either an elimination diet based on their true sensitivity results (specific diet) or a sham diet and followed for 6 weeks. 17 patients did not finish the trial.

disease was between 2 and 39 years (mean  $14.9 \pm 10.6$ ). Patients were excluded from participating if they had any significant co-existing disease. During screening some patients had to be excluded due to a lack of cooperation ( $n = 3$ ), severe concomitant disease ( $n = 10$ ), abscesses ( $n = 15$ ) or for C-reactive protein  $>150$  ( $n = 4$ ). Twenty-eight of the patients refused to participate. During the intervention phases the patients were allowed to take con-

comitant medication provided it had been constant for the 12 weeks of intervention (table 1). The constant medication over time was requested to determine a specific effect of nutritional intervention and not of higher doses of medication. This study was approved by the ethics committee of the University of Regensburg and performed according to the declaration of Helsinki. Informed consent was obtained from all patients.

**Table 1.** Baseline characteristics of the patients: 16 males (m) and 24 females (w) participated in the study

No.	Gender	Age	Disease localization	Treatment
1	m	21	terminal ileum, cecum	mesalazine, dimeticone, diltiazem
2	w	55	cecum to sigma	azathioprine, glibenclamide
3	w	24	colon, terminal ileum	budesonide
4	w	37	terminal ileum, cecum	budesonide, azathioprine, mesalazine, hydrocortisonacetate, loperamide
5	m	37	terminal ileum, proximal colon, proctitis	azathioprine, prednisolone
6	m	44	terminal ileum, cecum	azathioprine, prednisolone
7	m	41	ileum resection, multiple perianal fistulae	mesalazine
8	w	37	anastomosis	budesonide
9	m	37	distal ileum	azathioprine
10	m	27	terminal ileum, cecum	azathioprine
11	m	54	neoterminal ileum, colon, sigma, anal stenosis	cholestyramine
12	m	55	terminal ileum	azathioprine
13	w	31	esophagus lesions	infliximab, azathioprine, prednisolone
14	m	54	stenosis terminal ileum, ileocecal resection	none
15	w	57	sigma segment resection, stenosis colon descendens	cholestyramine
16	w	38	colon, sigma, rectum, stenosis terminal ileum	mesalazine, azathioprine, hydrocortisone
17	w	43	terminal ileum	azathioprine, azulfidine
18	w	54	colon	infliximab
19	w	54	colon	azathioprine
20	w	31	neoterminal ileum, colon	6-mercaptopurine
21	w	50	fistulae	azathioprine
22	w	33	neoterminal ileum	infliximab
23	w	25	colon, distal ileum	mesalazine
24	w	46	terminal ileum, caecum, sigma, rectum	budesonide, azathioprine
25	w	49	ileotransversostomy	azathioprine
26	w	57	fistulae	infliximab, azathioprine, azulfidine, mesalazine, cholestyramine
27	m	41	colon	prednisolone, azathioprine
28	w	48	colon, ileocecal resection	methotrexate
29	m	51	colon	budesonide
30	m	35	rectum	azathioprine, loperamide
31	m	29	colon	prednisolone, mesalazine, hydrocortisone
32	w	59	colon, neoterminal ileum	azathioprine
33	w	43	terminal ileum, colon	mesalazine
34	w	48	neoterminal ileum	budesonide
35	m	42	rectum, sigma, fistulae	prednisolone, ciprofloxacin, metronidazole
36	w	22	terminal ileum, colon	cholestyramine
37	w	28	colon	infliximab
38	w	44	terminal ileum, sigma	methotrexate, azathioprine
39	m	41	terminal ileum, colon	infliximab
40	m	35	colon	azathioprine

Patients were between 21 and 59 years of age ( $42 \pm 12$ ), treated with diverse drugs (treatment) and had different disease localization.

#### *ImuPro300 Test*

Sera from 40 patients was examined for food specific IgG by an enzyme-linked immunosorbent assay (ELISA) ImuPro300 test according to the manufacturer's recommendations (R-Biopharm, Darmstadt, Germany). Specific IgG antibodies against 271 food allergens (online suppl. table 1, for all online suppl. material see [www.karger.com/doi/10.1159/000264649](http://www.karger.com/doi/10.1159/000264649)) are possible to determine in human serum. The content of IgG antibodies is demon-

strated in table 2. Only high values (score 3) and very high values (score 4) of IgG were excluded from the diet of the patients. Diluted human serum was incubated in three different 96-well plates, each well coated with a different food extract. After washing the plates 3 times with diluted washing buffer, a polyclonal anti-human IgG antibody (sheep; R-Biopharm) conjugated to alkaline phosphatase was added. After washing with phosphate-buffered saline (PBS), substrate solution (pnpp, R-Biopharm) was

**Table 2.** IgG antibodies in patients' serum by ImuPro300

	IgG-class	Allergen-specific IgG content
<7.49 µg/ml	0 (0.0–0.9)	negative
7.5–12.49 µg/ml	1 (1.0–1.9)	weak
12.5–19.99 µg/ml	2 (2.0–2.9)	increased
20.0–49.99 µg/ml	3 (3.0–3.9)	high
≥50 µg/ml	4 (4.0–4.9)	very high

In patient-specific diets, only foods with score 3 (IgG content 20–49.49 µg/ml) and score 4 (IgG content ≥50 µg/ml) were excluded.

added to reveal the presence of IgG in the serum. Color development is proportional to the quantity of bound antibodies. After addition of a stop solution (NaOH; R-Biopharm), optical densities were measured photometrically (405/620 nm, Tecan Sunrise; Tecan GmbH, Crailsheim, Germany). IgG concentrations were calculated using a standard curve.

#### Collection of Peripheral Blood and Isolation of $T_{regs}$ and CD4+CD25- T Cells

50 ml of peripheral blood were obtained from each patient at the beginning of the trial and after 6 and 12 weeks. Blood was diluted with RPMI 1640 (Sigma-Aldrich Chemie, Steinheim, Germany) in a ratio of 1:2. Peripheral blood mononuclear cells (PBMCs) were isolated from the diluted blood by lymphocyte separation medium (PAA Laboratories GmbH, Pasching, Austria). 20 ml of lymphocyte separation medium were carefully covered with a layer of diluted blood and centrifuged at 400 g for 20 min at room temperature.

CD4+ T cells were isolated from PBMC using AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) with a CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions.  $0.4-1 \times 10^6$   $T_{regs}$  were isolated from 50 ml with the AutoMACS programs Depl05 and Pos-seld2. Normally,  $T_{regs}$  make up to 0.7–5.5% of PBMCs and 5–10% of T-helper cells [20, 23, 24]. CD4- cells in the remaining negative fraction were used to isolate antigen presenting cells (APC). CD4- cells were allowed to adhere to 96-well tissue culture plates (Falcon, Becton Dickinson, Heidelberg, Germany) for 2 h in an incubator at 37°C, 5% CO<sub>2</sub> (Heraeus 6000, Sepatech, Osterode, Germany). Nonadherent cells were removed by washing the wells repeatedly with prewarmed RPMI 1640. Adherent cells were used as APC.

#### Assay of Suppressor Function by $T_{regs}$

For suppression assays,  $0.5-1 \times 10^5$  CD4+CD25- T cells were cultured in the absence or presence of  $0.5-1 \times 10^5$  autologous  $T_{regs}$ /well in 96-well plates and in the presence of  $2 \times 10^5$  adherent APC in RPMI 1640 medium with 1% nonessential amino acids (100×) and 1 mM sodium pyruvate (PAA Laboratories GmbH, Pasching, Austria), 1% MEM vitamins (100×; Biochrom, Berlin, Germany), 25,000 U penicillin and 25 mg streptomycin (Gibco BRL Life Technologies, Eggenstein, Germany), β-mercaptoetha-

nol (final concentration  $3 \times 10^{-5}$  M; Gibco, Invitrogen, Karlsruhe, Germany), and 10% AB-serum (Cambrex Corporation, Europe). Cells were stimulated with food antigens (20 µg/ml; HAL Allergie GmbH, Düsseldorf, Germany), negative control solution (diluent without antigen, HAL Allergie GmbH) or Dynabeads® CD3/CD28 T Cell Expander (Dyna®l, Hamburg, Germany). These antigen solutions (as well as negative control solution) are commonly used for prick test analysis as previously described by Van Den Bogaerde [25]. The cells were cultured at 37°C, 5% CO<sub>2</sub> for 24 and 72 h, respectively.

#### Fluorescence-Activated Cell Sorting (FACS)

To determine the purity of the isolated T cell fractions, cells were stained with CD25-PE and CD4-FITC. The following antibodies were used: 10 µl of anti-human CD4-FITC, clone M-T466, isotype mouse IgG1 and 10 µl anti-human CD25-PE, clone 7D4, isotype mouse IgG2b (Miltenyi Biotec) according to the manufacturer's instructions. The antibodies were incubated for 15 min on ice and washed twice with PBS (PAA Laboratories GmbH). Subsequently, the cells were fixed with 3.7% paraformaldehyde, centrifuged at 300 g for 5 min and resuspended in 100 µl of PBS.

Stimulation of T cells with food antigens results in cell division with distinct fluorescence peaks, allowing determination of the number of cell divisions calculated by carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence (Sigma-Aldrich Chemie, Taufkirchen, Germany). CFSE is a fluorescein derivative which passively diffuses through the cell membrane and binds irreversibly to cytoplasmatic proteins. 24 and 72 h after in vitro stimulation, cells were analyzed by FACS. Proliferation is shown as a decrement of fluorescence because CFSE is distributed among the daughter cells. The cells were examined with an EPICS XL-MCL (Coulter Immunotech, Hamburg, Germany).

#### Determination of IFNγ

IFNγ secretion in cell supernatants was quantitatively measured by ELISA (IFNγ-ELISA Set; Biosciences, San Diego, Calif., USA) according to the manufacturer's protocol.

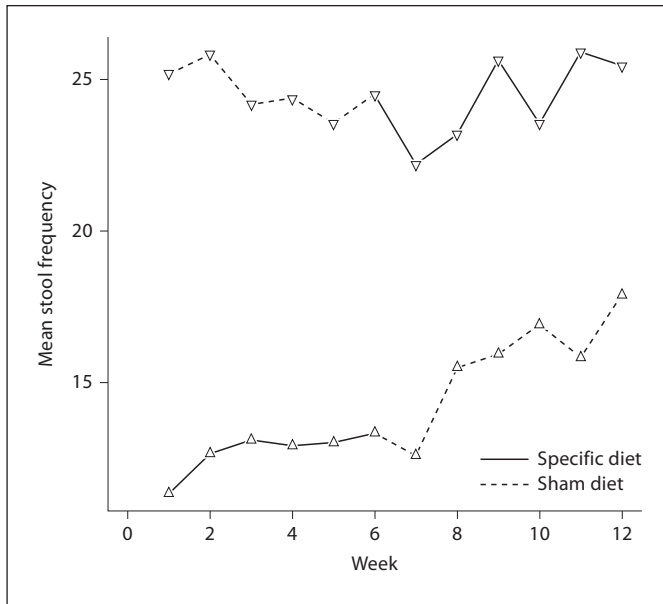
#### Determination of EDN

For the evaluation of a potential food allergy and disease activity, EDN was detected in 80 g of stool by ELISA (Immundiagnostik, Bensheim, Germany) according to the manufacturer's protocol.

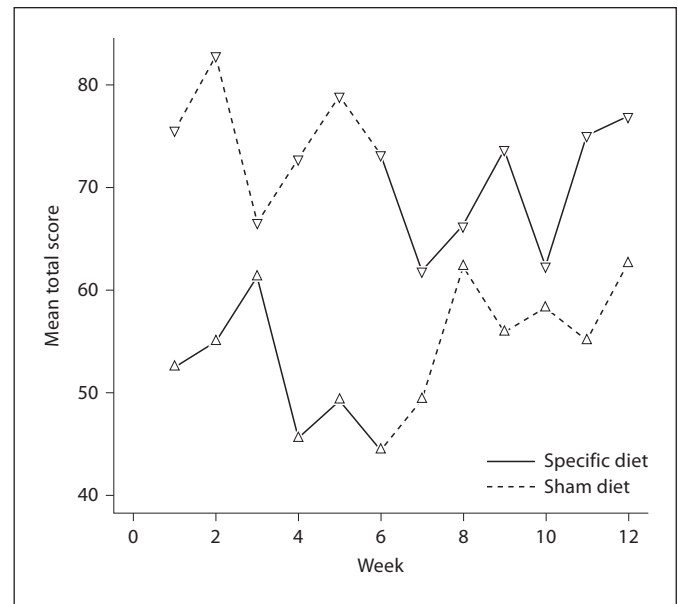
#### Data Analysis

Statistical analysis was carried out using SPSS, R and Sigma Stat. Weekly counts of stool frequency were analyzed with Poisson regression using generalized estimating equations (GEEs, [26]) to account for correlations between observations made from the same individual. This method provides a robust standard error for the treatment effect which was used to calculate confidence intervals and p values. Tests for cross-over effects were also performed by GEEs. Moreover, GEEs with normal outcomes have been used to analyze the total score. Data were not analyzed according to the intention-to-treat principle.

The application of all tests was verified by normality tests (Kolmogorov-Smirnov test, Shapiro-Wilk test). For statistical analysis of the pilot study, the quantity of patients and healthy controls with IgG antibody levels (in percent) was assessed by a t test. Statistical analysis of IFNγ secretion was performed by the



**Fig. 2.** Progression of stool frequency. Stool frequency was monitored per week. Only those patients who first followed the specific diet had a significant reduction in stool frequency. Subjects who first followed the sham diet had no significant change in their stool frequency (GEE analysis).



**Fig. 3.** Progression of total score. An average reduction of the total weekly score of 6.5 points was estimated for the specific diet group compared with the sham diet group (GEE analysis).

Kruskal-Wallis test based on non-normally distributed data. Values were significantly different when the obtained difference in mean ranks was greater than the  $\chi^2$  value (in all figures indicated with #). Values are expressed as the mean [minimum, maximum]. Statistical analysis of EDN in stool was performed by analysis of variance (ANOVA) for normally distributed data. Statistical significance was based on a p value smaller than 0.05.

## Results

### *Pilot Study-IgG Antibodies in CD Patients and Healthy Controls*

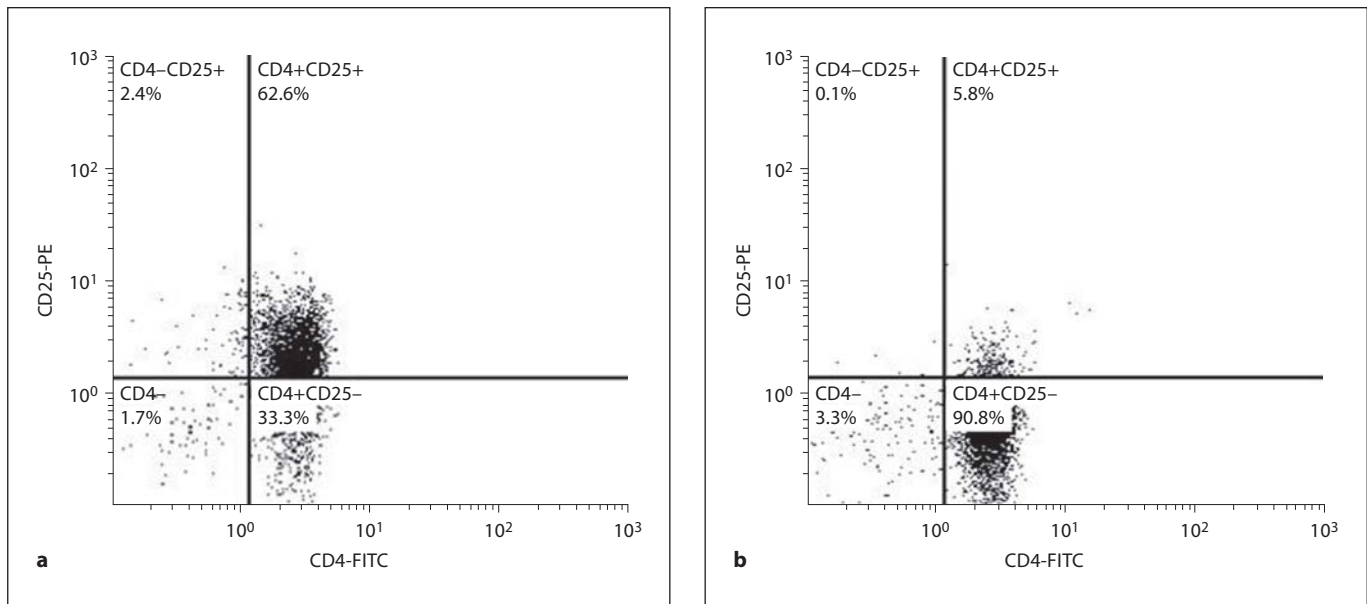
The pilot study resulted in a significant difference of IgG antibodies in serum between CD patients and healthy controls ( $p < 0.0001$ , t test). All detected IgG antibody reactions are presented online (online suppl. table 2). The ten most frequently measured IgG antibodies in CD patients were against processed cheese (84%), yeast (83%), agave syrup (78%), camembert cheese (76%), poppy seeds (74%), aloe vera (74%), bamboo sprouts (73%), kamut (durum wheat, 70%), unripe spelt grain (69%) and wheat (60%). More CD patients showed reactions against the evaluated food components than healthy controls, i.e. 35% of healthy controls had IgG antibodies against wheat in contrast to 60% of CD patients. Moreover, 39% of CD

patients had IgG antibodies against hazelnut in contrast to only 15% of healthy controls. This was even more pronounced in IgG antibodies against linseed, where 70% of CD patients and only 10% of healthy controls showed IgG antibodies. The same was seen with processed cheese (60% of healthy controls vs. 84% of CD patients).

The most frequently detected IgG antibodies in healthy controls were against yeast (66%), *Aspergillus niger* (60%), whey (60%), processed cheese (60%), bamboo sprouts (55%), paprika spice (55%), crawfish meat (50%), cottage cheese (45%), yoghurt (45%) and zander (45%).

### *Effects of the Nutritional Intervention on Stool Frequency, Abdominal Pain and General Well-Being*

There was no evidence for a cross-over effect in the analysis of the weekly stool frequency counts ( $p = 0.08$ ). In the specific diet group, a significant reduction in the daily stool frequency by 11% was achieved compared to the sham diet group ( $p = 0.004$ , 95% CI: 4%, 18%). However, the effect was confounded by a significant increase in stool frequency of 9% in the second intervention phase of the study, regardless of type of diet ( $p = 0.025$ , 95% CI: 1%, 18%; fig. 2). The comparison of loose stools during the specific and sham diets of each patient demonstrated that, surprisingly, only those patients who first followed



**Fig. 4.** FACS analysis of CD4+CD25<sup>-</sup> T cells and T<sub>regs</sub>. **a** Staining of T<sub>regs</sub>. **b** Staining of CD4+CD25<sup>-</sup> T cells (effector T cells). The purity of CD4+CD25<sup>-</sup> effector T cells was 90.8% and of T<sub>regs</sub> 62.6%.

the specific diet had a significant reduction in stool frequency. The group of subjects who first followed the sham diet and then specific diet had no significant change in their stool frequency.

Patients were asked to rate their pain perception and general well-being. The given points were accumulated after each week. To obtain an overall impression of stool frequency, abdominal pain and general well-being, a total score was calculated. There was neither evidence for a cross-over effect nor an intervention phase in the analysis of the total score. An average reduction of the total weekly score (fig. 3) of 6.5 points was estimated for the specific diet group compared with the sham diet group (95% CI: -0.6, 13.6 points). The estimated effect seems to have a clinically relevant effect, but is not significant ( $p = 0.07$ ).

#### *IFN $\gamma$ Secretion of CD4+CD25<sup>-</sup> Effector T Cells and T<sub>regs</sub>*

Isolation of CD4+CD25<sup>-</sup> effector T cells and T<sub>regs</sub> was controlled by FACS analysis. The purity of CD4+CD25<sup>-</sup> effector T cells was 90.8 and 62.6% for T<sub>regs</sub> (fig. 4a, b). CD4+CD25<sup>-</sup> effector T cells and APC were incubated in the absence or presence of T<sub>regs</sub> and stimulated with food antigens, negative control solution or antiCD3/antiCD28 solution. The effect of food antigens on CD4+CD25<sup>-</sup> T

cell activation was evaluated by quantification of IFN $\gamma$  in cell supernatants by ELISA (fig. 5–7). All obtained IFN $\gamma$  values were not normally distributed; therefore, a Kruskal-Wallis test was performed.

#### *Stimulation of CD4+CD25<sup>-</sup> and APC with Food Antigens and Negative Control Solution*

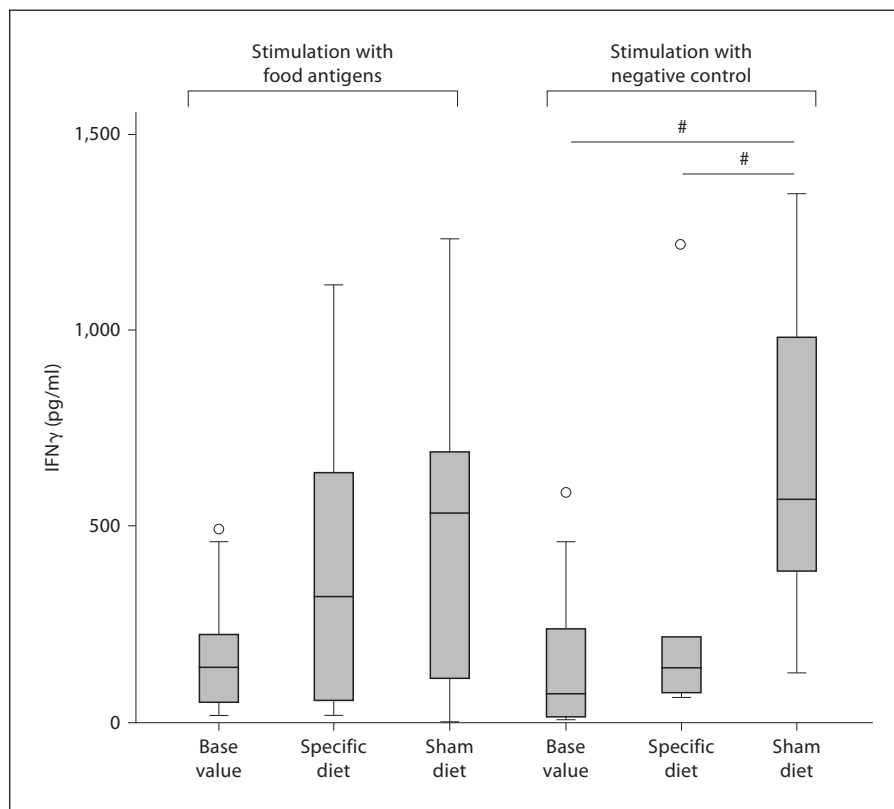
The incubation of CD4+CD25<sup>-</sup> T cells and APC with food antigens caused an increase in IFN $\gamma$  secretion (fig. 5, left panel). The amount of IFN $\gamma$  at base value (time point zero) was 170.5 pg/ml [16.5; 495.3] and increased during specific diet (411.1 pg/ml [16.9; 1,117.0]) and sham diet (481.5 pg/ml [1.5; 1,234.0]). Unfortunately, there was no significant difference between the three time points.

Stimulation of CD4+CD25<sup>-</sup> T cells and APC with negative control solution (fig. 5, right panel) showed IFN $\gamma$  secretion comparable to food antigen solution. The amount of IFN $\gamma$  at base value was 153.1 pg/ml [5.8; 587.6] and increased during specific diet (308.3 pg/ml [61.0; 1,220.0]) and sham diet (681.6 pg/ml [126.0; 1,347.0]). There was a significantly higher IFN $\gamma$  secretion during sham diet than during specific diet or base value, when cells were stimulated by negative control solution.

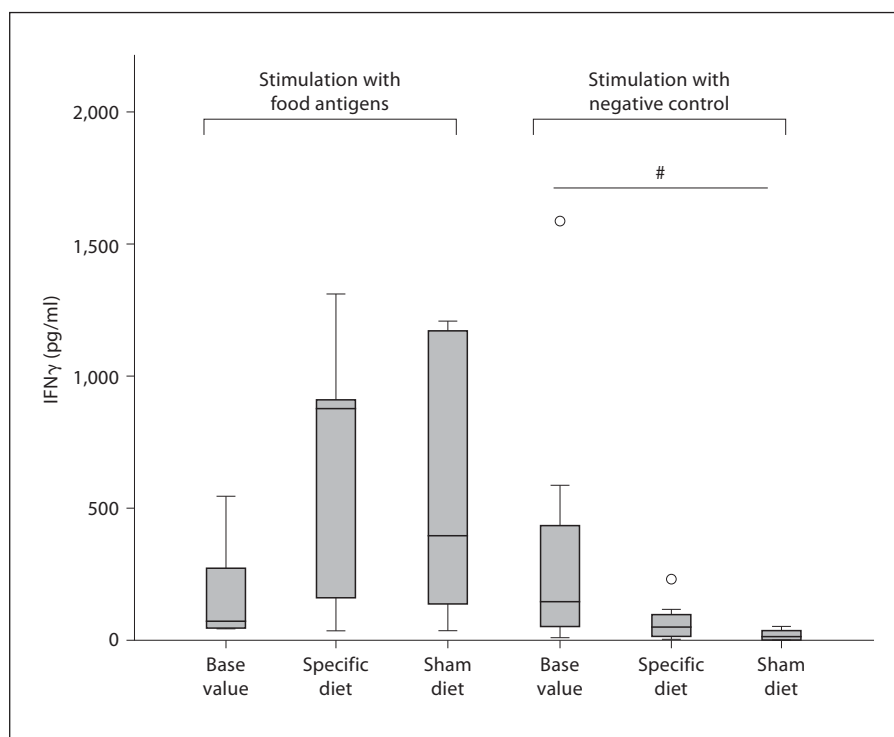
In summary, CD4+CD25<sup>-</sup> T cells were not clearly more stimulated by food antigen solution than by negative control solution.



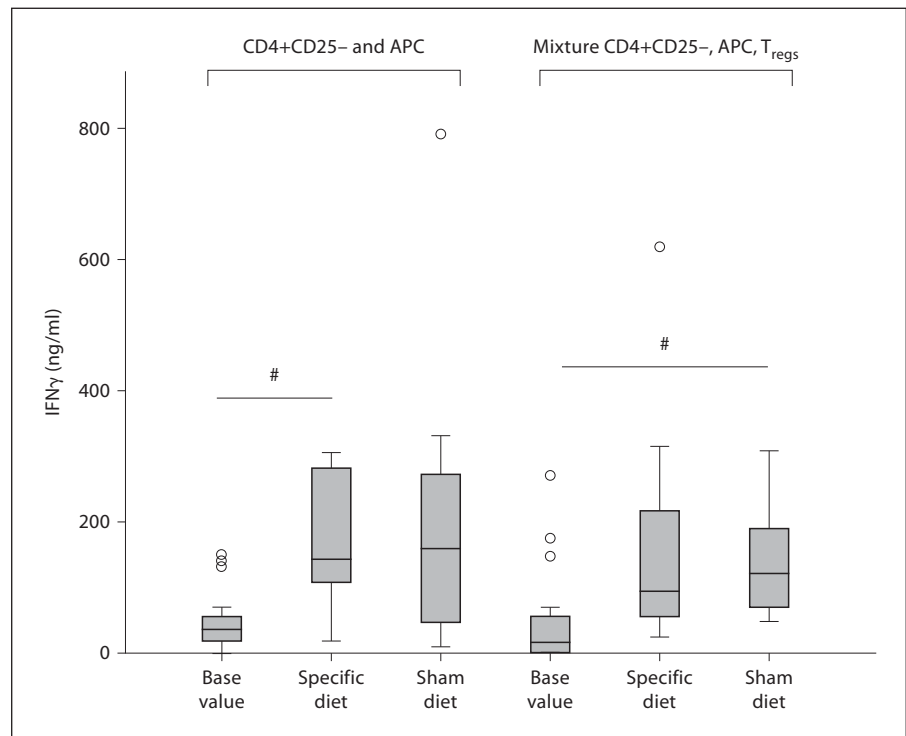
**Fig. 5.** Stimulation of CD4+CD25<sup>-</sup> T cells and APC with food antigens or negative control solution. Comparison of IFN $\gamma$  secretion (pg/ml) between base value (beginning of intervention), specific diet and sham diet (after 6 weeks of intervention, respectively). Stimulation with food antigens (left panel) increased IFN $\gamma$  secretion of CD4+CD25<sup>-</sup> T cells cultivated with APC during intervention. Stimulation with negative control solution (right panel) also increased IFN $\gamma$  secretion during intervention compared with food antigen stimulation. Kruskal-Wallis test; # indicates significance (obtained difference in mean ranks was greater than the  $\chi^2$  value);  $\circ$  indicates outliers.



**Fig. 6.** Stimulation of CD4+CD25<sup>-</sup> T cells and APC in the presence of T<sub>regs</sub> with food antigens or negative control solution. Comparison of IFN $\gamma$  secretion (pg/ml) between base value (beginning of intervention), specific diet and sham diet (after 6 weeks of intervention, respectively). Stimulation with food antigens (left panel): CD4+CD25<sup>-</sup> T cells co-cultivated with APC and T<sub>regs</sub> secreted more IFN $\gamma$  during specific diet and sham diet in contrast to base value. Stimulation with negative control solution (right panel): less IFN $\gamma$  secretion between base value and the specific and sham diets, respectively. Kruskal-Wallis test; # indicates significance (obtained difference in mean ranks was greater than the  $\chi^2$  value);  $\circ$  indicates outliers.



**Fig. 7.** Stimulation of CD4+CD25<sup>-</sup> T cells, APC and T<sub>regs</sub> with antiCD3/antiCD28 solution. Comparison of IFN $\gamma$  secretion (ng/ml) between base value (beginning of intervention), specific diet and sham diet (after 6 weeks of intervention, respectively). There was 1,000-fold higher IFN $\gamma$  secretion when cells were stimulated with antiCD3/antiCD28 solution than with food antigen/negative control solution. There was higher IFN $\gamma$  secretion of CD4+CD25<sup>-</sup> T cells (left panel) between base value and specific diet. The mixture of CD4+CD25<sup>-</sup> T cells, APC and T<sub>regs</sub> showed a significant increase only between base value and the sham diet. Kruskal-Wallis test; # indicates significance (obtained difference in mean ranks was greater than the  $\chi^2$  value);  $\circ$  indicates outliers.



#### Stimulation of the Co-Culture of CD4+CD25<sup>-</sup>, APC and T<sub>regs</sub> with Food Antigens and Negative Control Solution

The co-culture of CD4+CD25<sup>-</sup> T cells and APC with T<sub>regs</sub> and stimulation with food antigens (fig. 6, left panel) also resulted in an increase in IFN $\gamma$  secretion from base value (169.5 pg/ml [42.7; 543.1]) to specific diet (683.8 pg/ml [37.4; 1,311.0]) or sham diet (609.8 pg/ml [34.0; 1,209.0]). Unfortunately, there was no significant difference between the three time points.

There was lower IFN $\gamma$  secretion when co-cultivated CD4+CD25<sup>-</sup> T cells, APC and T<sub>regs</sub> were stimulated with negative control solution (fig. 6, right panel). Significantly lower IFN $\gamma$  secretion was only received between base value (294.4 pg/ml [10.1; 1,587.0]) and sham diet (21.2 pg/ml [1.9; 53.8]). Specific diet (71.6 pg/ml [2.9; 231.4]) resulted in no significant difference.

In summary, there was no clear difference between the culture of CD4+CD25<sup>-</sup> T cells and the culture of CD4+CD25<sup>-</sup> T cells with T<sub>regs</sub>.

#### Stimulation with AntiCD3/AntiCD28 Solution

The stimulation with antiCD3/antiCD28 beads of CD4+CD25<sup>-</sup> T cells and APC, or the co-cultivation of CD4+CD25<sup>-</sup> T cells, APC and T<sub>regs</sub> (fig. 7) showed high-

er IFN $\gamma$  secretion in contrast to the stimulation with food antigens or negative control solution. Therefore, cells were more effectively stimulated with antiCD3/antiCD28 solution than with food antigen/negative control solution, as seen in 1,000-fold higher IFN $\gamma$  secretion.

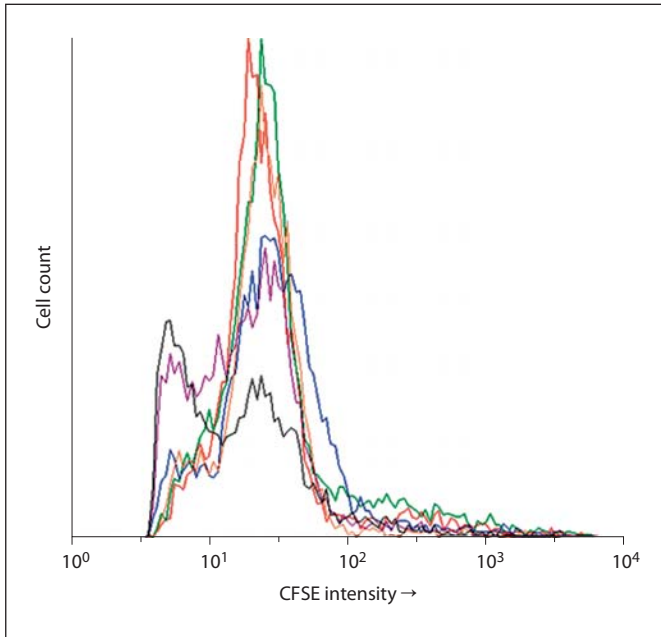
In case of CD4+CD25<sup>-</sup> T cells (fig. 7, left panel), a significant increase in IFN $\gamma$  secretion was detected between base value (48.2 ng/ml [0.8; 151.0]) and specific diet (169.3 ng/ml [19.6; 307.0]). There was no significant difference between base value and sham diet (204.6 ng/ml [12.10; 791.0]).

The mixture of CD4+CD25<sup>-</sup> T cells, APC and T<sub>regs</sub> (fig. 7, right panel) showed only a significant increase between base value (51.9 ng/ml [0.1; 271.0]) and sham diet (135.9 ng/ml [49.20; 309.0]). The increase during specific diet (157.9 ng/ml [26.20; 621.0]) was not significant.

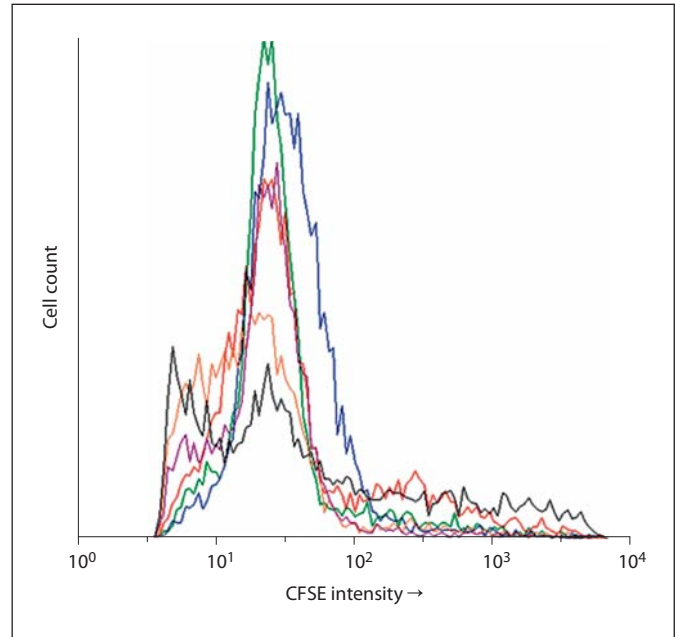
In summary, none of the three different stimulation methods led to a significant difference in IFN $\gamma$  secretion of CD4+CD25<sup>-</sup> T cells or the mixture of CD4+CD25<sup>-</sup> T cells and T<sub>regs</sub>.

#### Time Course of Cell Division after Cell Stimulation

The proliferative response of the isolated T lymphocytes to stimulation with food antigens, negative control solution and antiCD3/antiCD28 solution was investigated



**Fig. 8.** CFSE proliferation in the mixture of CD4+CD25- T cells, APC and T<sub>regs</sub> measured by FACS analysis. CD4+CD25- T cells and APC in the presence of T<sub>regs</sub> did not demonstrate any in vitro proliferative responses to food antigens. After 24 h: stimulation with food antigens (red), negative control solution (green) and antiCD3/antiCD28 solution (blue); after 72 h: stimulation with food antigens (orange), negative control solution (purple) and antiCD3/antiCD28 solution (black); graphs were recorded separately and are shown in overlay mode.



**Fig. 9.** CFSE proliferation of CD4+CD25- T cells and APC measured by FACS analysis. CD4+CD25- T cells and APC in the absence of T<sub>regs</sub> did not demonstrate any in vitro proliferative responses to food antigens. After 24 h: stimulation with food antigens (red), negative control solution (green) and antiCD3/antiCD28 solution (blue); after 72 h: stimulation with food antigens (orange), negative control solution (purple) and antiCD3/antiCD28 solution (black). Graphs were recorded separately and are shown in overlay mode.

by CFSE (fig. 8, 9). Each of the curves reflects a measurement of the fluorescence intensity caused by cell division. CD4+CD25- T cells and APC in the absence or presence of T<sub>regs</sub> did not demonstrate any in vitro proliferative responses to food antigens. Hence, the isolated T cells do not proliferate in vitro after stimulation with food antigens.

#### Quantification of EDN in Stool

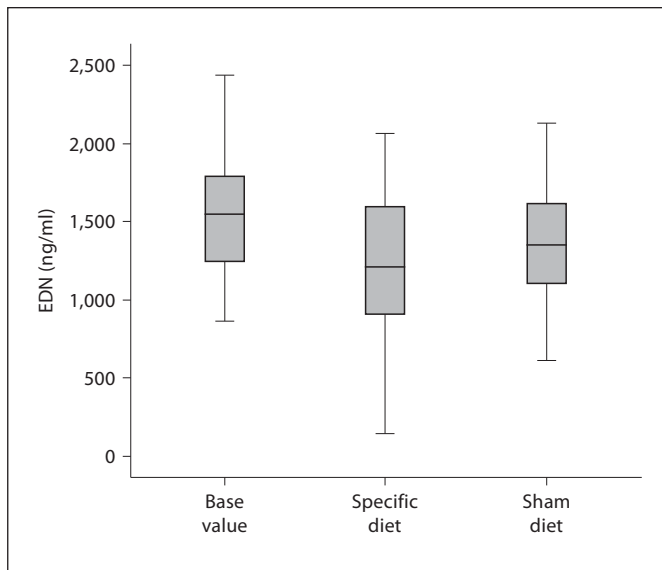
The disease activity was evaluated by the quantification of EDN in stool samples (fig. 10). The samples were normally distributed and, therefore, ANOVA was performed. The concentration of EDN at the beginning of intervention (base value) was  $1,536 \pm 405$  ng/ml. The concentration declined during specific diet ( $1,228 \pm 530$  ng/ml) but also during sham diet ( $1,355 \pm 373$  ng/ml). There was no significant difference between the three time points. EDN concentration dropped in the same degree under specific diet and sham diet. No difference for EDN in stool indicated an absence of eosinophil-mediated reactions.

#### Discussion

In the present study, we have shown that IgG antibodies against food antigens are elevated in patients with CD in contrast to healthy controls. A clinically significant improvement in IBD symptoms was observed in patients eliminating foods to which they were found to exhibit sensitivity. IFN $\gamma$  secretion by T cells was increased after specific diet, but also after sham diet. There was a reduction of EDN concentration in stool during specific diet and sham diet, but no significant difference between the two diets.

Forty-eight percent of patients in the present intervention study had an improvement in stool frequency and general well-being (total score). Only 9% of patients described opposite effects.

The study results are encouraging; however, they have to be interpreted with care as results may have been influenced by several confounders: The daily reporting of consumed foods and the attachment to the diet recom-



**Fig. 10.** Detection of EDN in stool samples. Comparison between base value (beginning of intervention), specific diet and sham diet (after 6 weeks of intervention, respectively). EDN concentration (ng/ml) declined during intervention in comparison to base value. There was no significant difference between the two diets. ANOVA test.

mentations required much time and discipline from the study subjects. Therefore, the problem of under-reporting (consumed foods and beverages were not listed correctly) is evident. We tried to reduce this problem by explaining the list of foods to be avoided in great detail to each patient and showing 'hidden sources'. This was done by one well-trained person.

Basically, the main limitation of this cross-over study was the high dropout rate ( $n = 17$ ), which was a result of the length of the study, the changes in CD and voluntary withdrawals.

In addition, the 40 patients initially included in this study were on different medications. Due to low numbers of individuals in this study, stratification according to the different treatments would have caused inability to do a statistical analysis. However, strong immunosuppressants certainly could have influenced the study results, especially with respect to T cell function. We tried to reduce this problem by keeping the medication constant for the time of intervention. In addition, many of the patients with CD already kept to some individual forms of diet, avoiding bloating foods such as onions or garlic [27–30].

In addition, it may be argued that the sham diet was too similar to the specific diet, as the definition of spe-

cific and sham diet was based on the similarity of excluded food components. If for example IgG against hazelnut was detected, then almond was excluded in the sham diet; if cauliflower IgG was found, broccoli was excluded. There may be some cross-reactivity of the respective antigens, which could explain some effects of the sham diet on IBD symptoms, T cell cytokine secretion and stool EDN levels.

In addition, we did not have a washout phase at the cross-over point, which may have led to some transmission of effects into the sham arm of the study.

More than 80% of CD patients in the pilot study and more than 30% of CD patients in the intervention study had IgG antibodies against yeast. The IgG-antibody reactions against food antigens were also investigated in CD patients by Van Den Bogaerde [25]. Increased sensitization against yeast was demonstrated in vivo and in vitro as in the present study. Additionally, a study from Darroch et al. [31] pointed out that antibodies against *Saccharomyces cerevisiae* are significantly increased in patients with CD in comparison to patients with ulcerative colitis and that they play a role in the function of T and B cells in patients with CD. In the present study, the difference in T cell function is shown by higher  $IFN\gamma$  secretion. Elevated  $IFN\gamma$  levels after nutritional intervention were detected in supernatants of cultures of  $CD4+CD25-$  T cells and APC in the presence or absence of  $T_{regs}$ . The mucosa of CD patients is dominated by T cells of the T-helper cell phenotype 1 [32].

These cells are characterized by the secretion of  $IFN\gamma$ . A greater number of cells secreting  $IFN\gamma$  in CD in contrast to ulcerative colitis and healthy controls was found [33].  $IFN\gamma$  is involved in specific cell-mediated immunity and causes the secretion of IgG2 antibodies during delayed-type hypersensitivity. Van Den Bogaerde et al. [25] illustrated, both in vitro and in vivo, a more pronounced reaction of T cells against food antigens. They tested the proliferation of peripheral blood lymphocytes after stimulation with different food antigens like cereal, cabbage, citrus fruits, yeast and nuts in 10 CD patients and 10 healthy controls [25]. They used commercial prick solution (just like in the present study) for cell stimulation. The authors concluded that in vivo sensitization against food antigens exists. The mechanism has to be further elucidated, perhaps due to a defective epithelial barrier function, which might allow infiltration of food antigens to the mucosa.  $IFN\gamma$  secretion of  $T_{regs}$  after stimulation with antiCD3/antiCD28 solution was also analyzed by Earle et al. [34]. In this issue,  $T_{regs}$  secreted no  $IFN\gamma$  in contrast to PBMC which secreted a concentration of 500

pg/ml. In addition, a co-cultivation of PBMC and T<sub>regs</sub> resulted in reduced IFN $\gamma$  production. In contrast to the findings of Earle, Nakamura et al. [35] postulated that T<sub>regs</sub> produce significantly less IFN $\gamma$  than CD4+CD25- T cells when stimulated adequately with antiCD3/antiCD28 solution. However, a limitation of the current study was that no FoxP3 staining was performed.

In the context of this study, a significant reduction of EDN concentration in stool during intervention could be detected. Values up to 1,300 ng/ml were examined. These findings are in line with results from Saitoh et al. [36] who found values up to 3,500 ng/ml in active CD and 910 ng/ml in inactive CD.

Similar to our data, an elimination diet in patients with irritable bowel syndrome was effective. One hundred fifty irritable bowel syndrome patients were tested and got a 'true' or a 'sham' diet. The true diet excluded foods against which IgG antibodies were found, the sham diet excluded foods against which no IgG antibodies were found (as in the present study). After 12 weeks of intervention, patients with the true diet had a 10% greater improvement of their symptoms than those patients with sham diet. Most of the patients within this study had IgG antibodies against yeast, followed by milk, chicken, egg, wheat and cashew nuts [37]. This study lacks a cross-over design in contrast to the present study. Moreover, immunohistochemical research found IgG producing B cells in the colon and ileum of CD patients [38, 39]. The authors

claimed that the raised IgG antibodies are due to a failure of intestinal barrier function. This circumstance could be one reason for the effectiveness of an IgG-based diet intervention.

In conclusion, a nutritional intervention diet based on circulating IgG antibodies against food antigens showed effects with respect to stool frequency, abdominal pain and general well-being in this double-blind cross-over study with 40 CD patients. Stool frequency and total score during the specific diet were significantly lower in contrast to the sham diet. Stimulation of T cells with the specific antigens was followed by an increase in IFN $\gamma$  secretion. However, there was no difference in T cell proliferation in response to different antigens. The concentration of EDN in stool declined during the specific diet, but also during sham diet. The mechanisms by which IgG antibodies might contribute to disease activity remain to be elucidated.

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