Mucosal Healing and Bacterial Composition in Response to Enteral Nutrition Vs Steroid-based Induction Therapy—A Randomised Prospective Clinical Trial in Children With Crohn’s Disease

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Abstract

Aims: Exclusive enteral nutrition [EEN] is as efficacious as corticosteroids [CS] to induce remission in Crohn’s disease [CD], without their adverse effects. EEN seems to be more efficient than steroids to induce mucosal healing, but the underlying molecular mechanisms are only sparsely understood. We aimed in the present work to study the anti-inflammatory effects of EEN with Modulen IBD® vs CS in active paediatric CD, and to assess its modulatory effects on the intestinal microbiota as compared with steroids.

Materials and Methods: Nineteen patients with new-onset active CD (Harvey-Bradshaw index [HBI] >5), aged from 6 to 17 years, were included in this prospective randomised induction trial with CS [n = 6] or EEN [n = 13]. Patients were assessed at Weeks 0 and 8 using clinical parameters HBI, endoscopic findings (Crohn’s Disease Endoscopic Index of Severity [CDEIS] score) and analysis of faecal microbiota composition.

Results: At 8 weeks, clinical remission [HBI <5] was achieved in 13/13 patients on EEN and 5/6 patients on steroids; the mucosal healing rate was significantly higher in the EEN [89%] compared with steroid group [17%]. There were no significant differences between groups regarding biological markers, but the intestinal microbiota profiles shifted upon EEN-induced remission to a higher proportion of Ruminococcus bacteria compared with steroid-induced remission [p = 0.049], and with higher proportions of bacteria belonging to Clostridium in EEN-treated patients.

Conclusions: Both steroid and EEN induced clinical remission. However, patients with EEN-induced remission showed a higher rate of mucosal healing and this was associated with a different gut microbiota compositional shift in these children.

Key Words: Crohn’s disease; exclusive enteral nutrition; microbiota
1. Introduction

Treatment strategies for Crohn’s disease (CD) evolved markedly over recent years for both adults and children. There is clear evidence that treatment efforts should go beyond the simple relief of acute symptoms, aiming complete control of inflammation with a long-term vision of improved outcome. In this sense, the paediatric committee of ECCO (PECCO) recently stated that the ultimate treatment target in children with CD should be a complete control of mucosal inflammation as reflected by mucosal healing, since this was shown to positively influence the natural evolution of the disease. There is clear evidence that obtaining complete mucosal healing is associated with fewer subsequent complications. The recent meta-analysis of Reinink et al. showed that complete [as well as partial] mucosal healing was significantly associated with improved outcome in adult patients with CD or ulcerative colitis (UC). Nuti et al. indicated in a prospective study that obtaining mucosal healing in children with CD improved outcome over the 24-month observation period. Steroid-free long-term remission is of utmost interest for this patients’ group characterized by a particularly severe disease presentation and exposed to several decades of disease evolution.

We wanted to take advantage of the potential of induction therapy with exclusive enteral nutrition (EEN) to induce mucosal healing, as opposed to corticosteroids (CS), and to further compare the gut microbial composition between patients achieving mucosal healing or not upon induction therapy. First studies report modifications of microbiota composition after EEN, paradoxically characterised by a reduced diversity and a decreased proportion of Bacteroides and ‘protective’ bacteria belonging to the Clostridium cocoides group. No data exist on the effect of steroids on microbial composition in CD patients, to our knowledge, and only scarce data focus on the link between intestinal microbiota and the achievement of mucosal healing. By designing a randomised clinical trial (RCT) in children with newly diagnosed CD, we addressed the question if patients in deep remission might differ in their microbial composition depending on the achievement of mucosal healing or not. This is of particular interest since a disturbed microbial composition (dysbiosis) is considered one main trigger of inflammation in patients with CD. Correction of dysbiosis is particularly attractive and might constitute an angle of attack upstream of the inflammatory cascade to develop new and more efficient treatment strategies for inflammatory bowel diseases. One might speculate that induction of remission by suppressing inflammation is one part of efficient therapy for CD, whereas correction of dysbiosis might add up to a more complete control seen in efficient mucosal healing. Induction therapy with EEN might be highly efficacious via a direct effect on the gut microbiome.

2. Materials and Methods

2.1. Study design

Children/adolescents with newly diagnosed CD [age range: 6–17 years] with active disease (Harvey-Bradshaw Index [HBI] >5) were enrolled in the present prospective randomised trial. Patients with ileocolonic or isolated small bowel disease [confirmed on endoscopy and imaging] were eligible after written consent of both parents and assent of the children [if applicable]. Exclusion criteria were: treatment by antibiotics during the 4 weeks preceding inclusion, corticosteroids, biologic therapies, immunosuppressive treatment, isolated oral or perianal disease location, risk of non-adherence to study protocol, and potential need for surgical therapy [abscess drainage, seton placement, or resection surgery for B2/B3 disease behaviour] during induction therapy. Patients were randomised to one of the two treatment groups [Cortancy® or MODULEN IBD®] initially planned at a 1:1 ratio, but due to low acceptance of the study protocol by the families [steroid avoidance], randomisation was amended to a 1 to 2 ratio [one in the CS group for 2 patients in the EEN group]. All inflammatory bowel disease (IBD) experts regularly using EEN for paediatric CD know that almost all parents are in favour of EEN, but that the acceptance rate of EEN by the patients themselves is markedly lower, further underlining the need to compare the two treatment options in several independent trials. Families who refused to use steroids were not eligible for this RCT. A total of 19 patients [13 EEN/6 steroids] were included over 36 months, with an overall acceptance rate of participation in this trial of 23%. The estimated energy requirements were calculated for all patients to define the optimal volume of EEN to be taken [calculation of basal caloric requirement plus additional needs for catch-up growth, usually 120% of theoretical caloric need]. EEN was given over an 8-week period as induction therapy. The dose of steroids was initially 1 mg/Kg per day [up to a maximum of 60 mg] during the induction period [4 weeks] and then tapered over 3 months. On steroid medication, patients had a free diet at libitum, a restricted sodium intake was advised, but a sodium-free diet was not recommended.

The primary outcome was defined as achievement of mucosal healing with a Crohn’s Disease Endoscopic Index of Severity [CDEIS] less than 3 points or a drop of >70% at follow-up endoscopy [8 weeks] compared with initial diagnostic endoscopy. All endoscopic examinations were performed by the same paediatric endoscopist blinded to the treatment arms. Secondary outcome parameters were clinical remission defined as a Harvey-Bradshaw Index [HBI] less than 5 and biological remission [mucosal and systemic inflammatory parameters]. The number of patients needed to be included was calculated to demonstrate a difference in obtaining mucosal healing between treatment with EEN and CS of at least 40%, with a type 1 error of risk of 5% and power 80%, giving a minimum of 6 vs 12 patients per treatment arms [ANOVA power analysis; repeated measures]. All patients provided faecal samples before, during, and at the end of induction therapy and during follow-up at 3, 6 and 12 months, in parallel with clinical and biological assessments.

2.2. Biological assessments

Mucosal biopsies obtained at colonoscopy from terminal ileum and sigmoid colon were snap-frozen in liquid nitrogen, and stored at -80 °C. Subsequently total RNA from biopsies was extracted using TRIZOL reagent [Life Technologies, Paisley, UK]. RNA quality was assessed by electrophoresis and RNA concentrations were determined by measuring the absorbance at 260 nm in a spectrophotometer; 1 µg of RNA was reverse transcribed in the presence of 500 ng oligo(dT) and 250 ng oligonucleotides with 1 µL reverse-transcriptase M-MLV, 1 µL RNaseOUT™ [40U/µL], 2 µL DTT 10 mM, and 1 µL dNTP 10 mM in 4 µL of 5X buffer [Invitrogen, Courtaboeuf France]. Samples were conserved at -20°C until analysis. Quantitative gene expression of IFNγ, IL-6, IL-10, IL-12, IL-17A, IL-17F, IL-23, and the housekeeping gene GAPDH was performed by real-time polymerase chain reaction [RT-PCR] using commercially available TaqMan gene assays [Applied Biosystems, Courtaboeuf France] according to established standard protocols. Then 40 cycles of amplification were performed with denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min using an ABI PRISM 7300.
PCR products were visualised on a 1% agarose gel [Sigma Chemical Co., St Louis, MO] stained with ethidium bromide [Sigma]. The intensity of bands’ brightness was measured using an image analyser under ultraviolet light [Seescan]. Ratios of the band intensities of the PCR products from the standard RNA and the target RNA allows quantification of the number of cytokine transcripts in a tissue sample down to as few as 10^2 and up to 10^5 transcripts per microgram of total RNA.

2.3. Intestinal microbiota analyses by fingerprinting and 454 pyrosequencing

Total DNA was extracted from faecal samples as previously described. DNA concentration and integrity were determined spectrophotometrically and bacterial 16S rRNA coding gene was amplified using primers targeting the V6-V8 region [GGClamp-U968 and L1401] for temporal temperature gradient gel electrophoresis [TTGE] fingerprinting and V3-V4 region for 454 pyrosequencing.

Sequence-specific fingerprints were obtained with TTGE as previously described. TTGE profiles were analysed with Gel Compar software version 2.0 [Applied Maths, Kortrijk, Belgium]. Similarity coefficients [Pearson correlation method] were calculated for each pair of profiles, yielding a similarity matrix. In-depth faecal microbial composition was further assessed by applying 454 pyrosequencing of the V3-V4 region of the 16S rRNA coding gene for eight patients over time [EEN n = 4; CS, n = 4]. Sequences were trimmed for barcodes and PCR primers, and binned for a minimal sequence length of 300 pb, a minimal base quality threshold of 27, and a maximum homopolymers length of 6. Resulting sequences were assigned to the different taxonomic levels, from phylum to genus using the RDP. 2.3. Data analysis

The results for continuous variables are given as mean standard deviation [s.d.], The results for non-continuous variables are given as frequency and percentage. Data at baseline and at follow-up were compared with the use of a Wilcoxon signed-rank test for matched pairs; p-values below 0.05 were considered significant.

For the analysis of microbiota fingerprints, dendrostromic curves corresponding to each of the normalised TTGE profiles were digitised with the GelCompar software program, from the migration distances ranging from 1 to 431 at steps of one interval and the observed optical density at each distance step. The resulting data matrix was used to assess microbiota modulation by either EEN or CS intake, and to calculate the spatial coordinates of each individual within a principal component analysis [PCA] using multivariate regression. PCAs were computed with the R software program [package ade4; http://pbil.univ-lyon1.fr/ADe4/]. In order to decipher the impact of the treatment on microbiota composition, interclass PCA with treatment as instrumental variable was computed based on the presence and abundance of each specific TTGE band; p-value of the statistical significance of interclass PCA clustering based on microbiota profiles was assessed using a Monte Carlo rank test [10 000 replicates]. For 16S rRNA gene sequencing, following reads trimming and analysis of 454 pyrosequencing data, a total of 32 800 sequences were further assigned to taxonomic levels for the eight selected patients, i.e. on average 2050 sequences per sampling time [Week 0 and Week 8] per patient. Wilcoxon testing was applied to assess statistical significance in bacterial composition modulation [at taxonomic levels of phylum, genus and OTUs] between the different samples and sampling time.

This clinical trial was registered under the number ClinicalTrials.gov Identifier: NCT00265772. This clinical trial received ethical approval for the Comité de Protection de Personnes [CPP] II Ile de France [no: 050911].

3. Results

A total of 19 patients were prospectively included in this study: 15 boys [79%] and four girls [21%]. The majority of patients were of Caucasian origin [18/19]. Median age at inclusion in this study was 12.3 years [range 7.5–15.8 years]. The demographic data at baseline are shown in Table 1. All patients had active disease at inclusion [HBI 5.0 ± 2.0 vs 6.7 ± 2.7; CS vs EEN allocation respectively, p = NS [non-significant]] with active ileocolonic inflammation on endoscopic evaluation [CDEIS: 10.4 ± 1.8 in the CS group compared with 7.4 ± 1.1 in the EEN group, p = NS].

3.1. Primary endpoint assessment

As per protocol, patients were randomised to receive induction therapy in the form of oral corticosteroids [Cortenyl® 1 mg/Kg, maximum 60 mg, for 4 weeks, followed by tapering] or in form of exclusive enteral nutrition [MODULEN IBD®] by mouth or by tube feeding. Follow-up endoscopy at Week 8 revealed mucosal healing in one of six patients [17%] in the CS group, whereas eight of nine patients [89%] on EEN induction therapy displayed complete mucosal healing, [p < 0.005 chi-square] [Table 2]. Four patients refused to have a follow-up endoscopy, since they felt well and did not perceive the reason to perform a follow-up procedure. On an intention-to-treat analysis, the mucosal healing rate in the EEN induction group was eight of 13 [62%], considering those patients who had no follow-up endoscopy as not achieving mucosal healing, thus resulting still in a clear, albeit non significant, difference between the CS and EEN groups [p-value = 0.068628 chi-square].

3.2. Secondary endpoint assessments

Disease activity as evaluated by the HBI showed a marked decrease in both treatment arms at Week 8, with a drop to HBI 0.1 ± 0.4 vs HBI 1.8 ± 2.2 in the EEN vs CS arm, respectively. Independently of the mode of induction therapy, all patients came into complete clinical remission at Week 8 in the EEN group, and all except one patient in the CS arm.

Inflammatory parameters, such as C-reactive protein [CRP] and erythrocyte sedimentation rate [ESR], dropped significantly in both treatment arms [Table 2], with no significant difference between the two groups.

3.3. Cytokines profiles

Analysis of mucosal cytokine expression was only possible in a subgroup of 11 patients [eight in the EEN group and three in the CS group]. In EEN-treated patients, a clear drop of inflammatory parameters, especially IFN and IL17, was observed within the intestinal mucosa, by RT-PCR analyses [Table 3]. However, this did not reach statistical significance. Similarly, in CS-treated patients, inflammatory parameters decreased. When comparing the two treatment groups at Week 8 for IFN, IL17, IL12, IL22, and HBD2, no
A significant difference was observed between CS- or EEN-induced remission.

### 3.4. Microbiota composition in EEN- vs CS-treated patients.

Applying fingerprinting analysis of the dominant faecal microbiota to all 19 patients before, during, and after treatment, a significant clustering was observed between microbiota of patients at Weeks 0 and 8. Microbial modulation was significantly different depending on the mode of induction therapy. Interclass principal components analysis showed a significant clustering [Monte-Carlo rank test $p = 0.049$] before treatment, and during CS or EEN treatment, regarding dominant microbiota composition [Figure 1]. Yet, no specific fingerprinting band was sufficient to explain and resume the observed differences between EEN- or CS-treated patients' microbiota, and a pyrosequencing approach was applied on a subgroup of eight patients [four patients in each treatment arm, at Week 0 and Week 8].

**Table 2. Characteristics of the EEN and CS groups at baseline [Week 0]**

<table>
<thead>
<tr>
<th></th>
<th>EEN group [n = 13]</th>
<th>CS group [n = 6]</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female ratio</td>
<td>3/13</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Age [years]</td>
<td>11.7 ± 2.5</td>
<td>13.7 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>BMI [Kg/m²]</td>
<td>14.5 ± 1.2</td>
<td>17.8 ± 3.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>z-score BMI</td>
<td>-1.77 ± 0.9</td>
<td>-0.55 ± 1.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Harvey-Bradshaw Index [means]</td>
<td>6.7 ± 2.7</td>
<td>5.0 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Haemoglobin [g/dL]</td>
<td>10.3 ± 1.0</td>
<td>11.2 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>CRP [mg/L]</td>
<td>10.3 [8.0–12.0]</td>
<td>11 [10.5–12.0]</td>
<td>NS</td>
</tr>
<tr>
<td>ESR [mm]</td>
<td>50 [7–100]</td>
<td>65.5 [11–76]</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin [g/L]</td>
<td>44.7 ± 18.5</td>
<td>29.6 ± 17.8</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet counts [mm³] [means]</td>
<td>27.6 ± 4.3</td>
<td>33.5 ± 10.5</td>
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<tr>
<td>CDEIS ≤3</td>
<td>567769</td>
<td>456167</td>
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<td>CRP [mg/L]</td>
<td>12.3 ± 19.3</td>
<td>42.8 ± 74.8</td>
<td>NS</td>
</tr>
<tr>
<td>Median</td>
<td>6 [1–75]</td>
<td>14 [6–195]</td>
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<tr>
<td>ESR [mm]</td>
<td>16.5 ± 24.5</td>
<td>21.2 ± 23.7</td>
<td>NS</td>
</tr>
<tr>
<td>Median</td>
<td>8 [4–94]</td>
<td>12 [7–69]</td>
<td>NS</td>
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<tr>
<td>Albumin [g/L]</td>
<td>38.7 ± 5.8</td>
<td>37.6 ± 4.0</td>
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<tr>
<td>Platelet counts [mm³]</td>
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</tr>
<tr>
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<td>394833</td>
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</tr>
</tbody>
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EEN, exclusive enteral nutrition; CS, corticosteroid; BMI, body mass index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; CDEIS, Crohn's Disease Endoscopic Index of Severity; NS, non-significant.

At Week 0 [diagnosis of CD], no significant differences in bacterial composition were observed between both randomly assigned groups. It is noteworthy that at the phylum level, no specific dysbiosis was observed in the eight new-onset CD patients before any medication, before starting either EEN or CS. Main phyla related to Firmicutes [61%], Bacteroidetes [33%], Actinobacteria [3%], and Proteobacteria [1%]. Main genera for each patient at Week 0 are represented in Figure 2 and were individual-specific.

Following EEN treatment, patients' microbiota were largely enriched in bacteria from the genus Clostridium XIVa, but markedly depleted in bacteria from the Faecalibacterium and Roseburia genera, when compared with CS-treated patients' microbiota at 8 weeks [Figure 3]. On the other hand, in CS-treated patients, pyrosequencing unravelled an enrichment of Ruminococcus and decreased proportions of bacteria from the Blautia genus [8.66% at Week 0 vs 5.22% at Week 8; paired t test $p = 0.044$]. A trend in increased Bifidobacterium proportions was observed at the end of CS therapy [1.60% vs 4.94%; paired t test $p = 0.057$].
Finally, at a species level, EEN treatment led to a significant increase in proportions of several bacteria from the Clostridium, mainly clusters XIVa and IV [Figure 4B]: Clostridium symbiosum and C. ruminantium, as well as Ruminococcus torques (Week [W0]: 0.1% to W8: 2.24%), Ruminococcus gnavus [W0: 0.12% to W8: 6.32%], Clostridium hathewayi [W0: 0.1–W8: 7.2%]. At W8, only steroid-treated patients displayed enrichment in butyrate producers, such as bacterium M62, A186, Roseburia intestinalis and Eubacterium, and steroid induction therapy led also to enrichment in Bifidobacterium bifidum [Figure 4A and B].

The microbial diversity [Shannon’s index and/or Simpson and number of observed OTUs] tended to increase after EEN therapy with Modulen from 3.82 to 5.0, whereas on steroid medication the change was only minimal, from 5.39 to 5.75. Since we have only four children in each group, it is not possible to conclude and compare the efficacy of treatment based on these data.

### Table 3. Comparison of cytokines levels in EEN-treated patients between Week 0 and Week 8

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Week 0 Mean ± SD</th>
<th>Week 8 Mean ± SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL17</td>
<td>88.1 ± 32.3</td>
<td>29.1 ± 9.7</td>
<td>0.08</td>
</tr>
<tr>
<td>IFNg</td>
<td>539.4 ± 192.9</td>
<td>102.9 ± 41.1</td>
<td>0.06</td>
</tr>
<tr>
<td>IL23</td>
<td>182.3 ± 97.6</td>
<td>190.1 ± 113.1</td>
<td>NS</td>
</tr>
<tr>
<td>IL12</td>
<td>171.5 ± 56.8</td>
<td>82.1 ± 16.6</td>
<td>NS</td>
</tr>
<tr>
<td>IL22</td>
<td>139.1 ± 88.4</td>
<td>34.0 ± 11.4</td>
<td>NS</td>
</tr>
<tr>
<td>HBD2</td>
<td>970.1 ± 876.9</td>
<td>142.2 ± 128.9</td>
<td>NS</td>
</tr>
<tr>
<td>IL10</td>
<td>532.9 ± 174.9</td>
<td>350.2 ± 105.1</td>
<td>NS</td>
</tr>
<tr>
<td>IL6</td>
<td>79.1 ± 42.7</td>
<td>21.3 ± 9.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

EEN, exclusive enteral nutrition; NS, non-significant.

### 3.5. Correlations between bacterial genera and clinical or blood parameters

The genus Ruminococcus was positively correlated with body mass index \( r = 0.68 \) as well as with haemoglobin levels \( r = 0.62 \) and albumin \( r = 0.56 \). Sedimentation rate was negatively correlated with both Eubacterium \( r = -0.5 \) and Hepsellia \( r = -0.5 \). On the other hand, unassigned bacterial sequences were negatively correlated with both CRP \( r = -0.76 \) and albumin \( r = -0.73 \) [Figure 5].

### 4. Discussion

This randomised clinical trial in paediatric CD was designed to measure mucosal healing [primary outcome] upon standard induction therapy [steroid vs EEN] with a particular attention to changes in the microbial composition. It is well established that both treatment options allow induction of remission in the majority of CD patients; the results of the present study are in line with the trial of Borrelli and colleagues,18 and also with the results from the paediatric meta-analyses of Heuschkel19 or Dziecharz,20 which both highlighted equal efficacy of either induction therapy. Even if clinical and inflammatory parameters are improving under both treatment options, mucosal inflammation still can persist, as clearly demonstrated in the present study in patients receiving steroids as well as in the prospective RCT of Borrelli et al.18 In the Italian study,18 a combined histological and endoscopic score [CDEIS] was used to evaluate the potential of inducing mucosal healing [MH] on steroid vs enteral nutrition therapy. Comparing 19EN- with 17 CS-treated patients, a mucosal healing rate of 74% vs 33% in EN- vs CS-treated patients was observed. A second retrospective study on 37 children on EN vs 10 children receiving steroids, revealed also a significant albeit smaller difference in MH rates of 25% favouring EN.21 Surprisingly, our study observed an even more important difference in obtaining mucosal healing, strongly favouring EN; when corrected to an intention-to
treat-analysis, this difference dropped to 45%, which is in the same range as the Italian prospective RCT reporting 41% of healing mucosal lesions. The MH rate in our study in children receiving steroids is probably underestimating the true rate. This is probably due to the study design: the very limited number of patients and complete successful follow-up endoscopic examinations. This does not allow firm conclusions on the absolute numbers and statistics; RCTs based on small numbers of patients are more vulnerable to potential bias compared with largely-sampled multicentre studies. Nevertheless, the present data confirm a clear trend in keeping with previous studies, and it allowed subsequent qualitative analysis. Mucosal healing is of utmost importance, since the success for maintenance of remission

Figure 2. Relative abundance of main bacterial genera distribution for each of eight patients included in the sub-cohort (four patients treated with EEN and four patients treated with steroids) at Week 0. EEN, exclusive enteral nutrition.

Figure 3. Over time modifications of specific bacterial genera before starting treatment (Week [W] 0), during exclusive Modulen treatment [W8 EEN] and during corticosteroid treatment [W8 CS]. Data are expressed as relative abundance [percentages]. Only main genera with significant differences between groups are presented [Wilcoxon p-value <0.05]. EEN, exclusive enteral nutrition; CS, corticosteroid.
Figure 4. (A) Heatmap of the main bacterial species isolates [Best RDP Sab score affiliation of OTUs with any bacterial isolates] shifting between the three different conditions [before treatment, during CS, and during EEN treatment]. Data were normalised as z-score. Red indicates low z-score and low relative abundance, and yellow-white stands for bacterial species with higher relative abundance. (B) Boxplots of bacterial species significantly different between W0 and W8 in either the EEN or the CS group. EEN, exclusive enteral nutrition; CS, corticosteroid; OTU, operational taxonomic unit.
with immunomodulators, such as thiopurines, relies markedly on the
efficacy of induction therapy. Based on our data and the study of
Borelli et al., showing that EEN has a markedly higher potential
to induce mucosal healing than steroid medication, it is not surpris-
ing that maintenance of remission with thiopurines is significantly
higher in patients receiving EEN as induction therapy compared with
steroid-induced remission, as recently indicated by Grover et al. and
our own data [Chouchana et al. submitted].

Yet, the mechanism of EEN action remains unclear, but one
main hypothesis is that EEN, via a modulation of gut microbiota
and correction of dysbiosis, may shift the microbiota towards an
anti-inflammatory profile. An unbalanced intestinal microbiota, i.e.
dysbiosis, has been repeatedly pointed out in IBD and is now recog-
nised as a major key actor in the gut inflammatory process. From
a therapeutic point of view, the correction of the dysbiosis is thus an
attractive approach in IBD.

In the present study, induction of clinical remission led to a
significant shift in the microbial composition of patients in both
treatment arms at Week 8. Even though both treatments led to
enrichment in bacterial species belonging to the Firmicutes phy-
lum, microbial modulation was significantly different depending on
the mode of induction therapy. Specifically, Ruminococcus gnavus,
Ruminococcus torques, and several Clostridium species were signifi-
cantly more important following EEN. Both R. gnavus and R. tor-
ques belong to Clostridium XIVa, but in contrast to other members
of this group, they are not butyrate-producers but are described as
mucolytic bacteria. Both have been highlighted in CD, either in
patients or in their unaffected relatives. Whether their abundance is
associated with inflammatory parameters or a different mucus pro-
file or structure in IBD, or may reflect increased mucus production,
remains unclear to date. Our results are in keeping with the results
of Quinse et al., who described a decrease in Bifidobacterium,
Faecalibacterium prausnitzii, and an increase in R. gnavus and
Enterococcus faecalis in EEN. EEN has also been associated with a
decrease in butyrate production.

CD-associated dysbiosis is now well described. Sokol et al. showed
a significant decrease in the proportion of the Clostridium leptum
[Cluster IV] phylogenetic group in patients with colonic CD. These
results were confirmed by a metagenomic approach revealing a re-
striction in biodiversity within bacteria belonging to Clostridium IV
and Clostridium XIVa groups. Using a clinically-based rational se-
lection process, Sokol et al. identified Faecalibacterium prausnitzii [a
major bacterium of the Clostridium IV group] as a key actor in intes-
tinal homeostasis, with therapeutic potential in IBD. In adult IBD
patients, all studies reported a decrease in Faecalibacterium praus-
nitzii proportions. In contrast, children with CD exhibit substantial
percentages of F. prausnitzii. Russell et al. even reported an increased
load of F. prausnitzii in ileal biopsies of new-onset paediatric CD
patients. In the present study, we did not observe a loss of F. praus-
nitzii proportions at inclusion and no clear dysbiosis could be identi-
cified. This finding is in contrast to the microbial analyses in adult CD
patients, and might be either related to the age of disease onset or,
more likely, to the fact that the paediatric cohorts included patients
at diagnosis and most often before starting therapy. Paradoxically,
we observed lower percentages of F. prausnitzii in patients in remis-
sion after EEN treatment, contrasting with those receiving steroids.
This is in line with the recent findings of Gerasimidis: comparing
15 children with CD to 21 controls, a global decrease of the bacterial
diversity was observed along with a decrease in F. prausnitzii. This,
as well as our study, highlights the finding that dysbiosis in children
with CD is different from dysbiosis observed in adult CD. One might
hypothesise that Faecalibacterium prausnitzii depletion is a marker
of a prolonged dysbiosis, or that this bacterium is less important in
the gut homeostasis of children.

Based on a small group of children with CD [11 newly diagnosed
and 10 patients already on IBD medication], Gerasimidis et al. iden-
tified a decrease of bacterial diversity after 8 weeks of EEN. In
the present study, we tried to mimic these findings based on samples
of four patients. However, given the fact of an extremely poor bacterial
diversity at diagnosis [before any medical therapy] in our patients
with active inflammation, we were not able to reproduce these find-
ings. We saw a slight increase of bacterial diversity during EEN,
wheras patients on steroids had no change of diversity. However,
it is to be expected that in general a restricted diet [such as used in
this study] reduces diversity, as shown by Gerasimidis, At the current
stage, it is not possible to extrapolate our data based on only four
patients. It is of interest to gain further insight into the bacterial
diversity of children, especially at young age with IBD before starting
therapy, since adult data cannot be easily extrapolated to the situa-
tion of pediatric IBD.

Our present study highlights that EEN leads to a decrease in propor-
tion of potentially beneficial bacteria. Therefore, regarding the
microbiota modifications induced by EEN and the clinical
improvement and mucosal healing observed, it is difficult to explain
the anti-inflammatory action of EEN exclusively by the modula-
tion of gut microbiota. In fact the mechanisms of action of EEN are far
more complex than just changes in microbiota composition. Several
hypotheses were proposed to explain the anti-inflammatory poten-
tial of EEN. Some authors highlighted the role of dietary antigens
and the reduced antigenicity hypothesis. EEN profoundly reduces
the amount of food antigens, decreasing thereby the stimulation of
the immune system by nutrients. Even if formulas with fully hydrol-
ysed proteins or elemental diets are no more effective than partially
hydrolysed formulas or formulas with intact proteins, a diet with

Figure 5. Correlation between bacterial genus and clinical/blood parameters.
a single protein source contains far less antigens than a usual diet. Therefore, some components of table food could contribute to the inflammatory process in patients with CD.

EEN also reduces the supply of exogenous bacteria and bacterial components contained in a normal diet. Dietary factors may serve as ligands for various host receptors. For example, the aryl hydrocarbon receptor [AHR] is expressed on intestinal dendritic cells and lymphocytes and helps maintain epithelial integrity. AHR has many ligands, some of which are present in food. Toll-like receptors 2 and 4 are activated by saturated fatty acids but inhibited by omega-3 polyunsaturated fatty acids [PUFAs]. EEN, by reducing the amount of specific factors in food, may therefore have effects on mucosal integrity and immune function.

The last hypothesis is that EEN acts by modulating the gut cytokines profile. Breese et al. demonstrated that enteral nutrition reduced the number of mucosal cells producing interleukin-2, interferon, and TNF-α. It was shown that EEN stimulated TGF-β1 production in CD patients, and MODULEN IBD®, used in this study, is enriched in TGF-β2. Thus anti-inflammatory effects of EEN may be related to its direct impact on the inflammatory cascade at the intestinal mucosal level with TGFβ as particularly potent and central mediator, enhancing epithelial restitution and wound healing. TGFβ is a potent anti-inflammatory cytokine; studies in murine models of inflammation clearly showed that elimination of TGFβ or disruption of its downstream signalling cascades caused chronic inflammatory lesions. Similarly, transgenic mice with inactivated TGFβ signalling develop spontaneous colitis. More recently, Nguyen et al. demonstrated that TGF-β2 of dietary or endogenous origin regulates the intestinal epithelial cells responses against lipopolysaccharide stimuli, thereby supporting cellular homeostasis and innate immunity in response to bacterial colonisation.

As indicated, our study has several limitations, especially the small number of patients included. This reflects the difficulty in performing such a trial in children/adolescents with CD, who were quite reluctant to repeat endoscopy and, in some families, also to receive steroid medication considered as subsitomial choice. It hampered studying associations between the effects of EEN on CDEIS or mucosal healing and the gut microbiota composition [only one child with mucosal healing in the Cs group, and only one without in EEN group]. Sokol et al. described Faecalibacterium prausnitzii as a marker of clinical remission after surgical resection, introducing the idea that some bacterial species could be good prognostic markers in IBD. The group of Van Limbergen et al. described microbial community structure in healthy children and in CD patients before and after EEN, allowing the identification of some species as markers of sustained remission after EEN treatment. The authors tried to propose a novel approach of microbiota analysis at diagnosis in order to predict sustained remission. We were not able in the present study to confirm these findings, probably due to the small number of patients analysed herein. However, in the future, microbiota description at diagnosis might be an interesting basis for individualised therapy.

In summary, nutritional therapy [EEN] is a highly attractive treatment option for CD whether by oral or by tube feeding. Our recent ECCO/ESPghan Guidelines stated that EEN is the first-choice induction therapy for children/adolescents with active luminal disease, and EEN is used in many specialist centres in Europe as standard therapy. The mechanism[s] of action of nutritional therapy are not yet completely understood. Nevertheless, current evidence shows that direct anti-inflammatory effects, mucosal healing, and modulation of the intestinal microbiota contribute to the therapeutic properties of EEN.

Funding
The present work was supported by a non-restricted grant of Nestle France.

Conflict of Interest
OG received financial support for research from Danone, Fresenius Kabi, Biocodex, Shire. JD received financial support for research from Danone, fees for lecture and editorial work from Jansen and Biocodex, consultancy and shares as co-founder of Enterome and MaasT Pharma. FR has received research supports from Nestle Nutrition Institute, AbbVie, MSD, Jansen and Jansen, and lecture fees from AbbVie, Danone, Nutricia, Nestlé, and served as member of advisory board: DEVELOP [Centocor], CAPE [AbbVie], LEA [AbbVie], SAC for MSD France, Nestlé Nutrition Institute, Nestlé Health Science, Danone, MeadJohnson; Nutricia, Takeda, Celgene, Biogen, Shire, Pfizer, Therakos.

Acknowledgments
The authors acknowledge the excellent technical skill of Florence Levenez performing the experimental analyses. We also wish to thank the motivation and generosity of the patients and their parents to participate in this somewhat difficult clinical trial.

Author Contributions
FR and JS designed the study; BP, FR, OG, and JS enrolled the patients; PL, SM, and JD performed TTGE and pyrosequencing analysing; BP, PL, and FR reviewed and analysed data; BP, FR, and PL wrote the manuscript, which was reviewed by all authors.

References
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