Lipidomics reveals significant alterations associated with exclusive enteral nutrition treatment in adult patients with active Crohn's disease

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Background: Crohn's disease (CD), a type of inflammatory bowel disease, is a chronic idiopathic disorder of the gastrointestinal tract with an increasing global incidence. Exclusive enteral nutrition (EEN) is a diet therapy that is effective in the management of active CD with unknown etiology. Lipid metabolism plays an important role in CD and may be associated with EEN treatment. This study compared the plasma lipid profiles before and after EEN in adults with active CD to those of healthy controls (HCs).

Methods: Eleven adult patients with active CD who received enteral nutrition formula treatment for 12 weeks were included, along with 17 HCs. The profiles of 869 plasma lipid species were measured, and inflammatory and nutrition-associated indices were evaluated in the patients.

Results: Nine patients achieved clinical remission following 12 weeks of EEN treatment, and four achieved mucosal healing. Before EEN, 80 lipid species and 17 lipid classes were significantly different between patients with CD and HCs. After EEN treatment, 103 lipid species and 12 lipid classes were significantly different between patients with CD and HCs. Significant changes in 7 lipid classes and 38 lipid species were observed between the pre- *vs.* post-treatment CD patients. The levels of simplified glucosylceramide series, monogalactosyldiacylglycerol, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine increased, while those of phosphatidylglycerol and phosphatidylinositol diphosphate decreased significantly after EEN. These lipid classes and species were associated with the inflammatory and nutritional indices. Pathway analysis suggested the metabolism of arachidonic acid, glycerophospholipids, linoleate, and phosphatidylinositol phosphate was related to the EEN mechanism.

Conclusions: EEN induces alterations in multiple lipid classes and species, leading to clinical improvements. Lipid metabolism may be involved in the EEN anti-inflammatory effect.

Keywords: Crohn's disease (CD); exclusive enteral nutrition (EEN); lipid profile; ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS)

Introduction

Crohn's disease (CD) is an inflammatory bowel disease (IBD), which represents a group of progressive disorders with recurrent chronic intestinal inflammation. Diarrhea, abdominal pain, rectal bleeding, fever, weight loss, exhaustion, growth retardation, anemia, recurring fistulas, or extraintestinal manifestations are typical CD symptoms (1). A transmural, discontinuous inflammatory process, along with focal crypt abnormalities and non-caseous epithelioid granulomas, are revealed by CD histopathology (2). Over the past three decades, the prevalence of CD has increased by almost 40 times worldwide. The incidence is higher in Europe and North America (3.77 to 6.38 cases per 100,000 person-years) and is rising worldwide, particularly in areas with previously low morbidity (Africa, Asia, and South America) (3,4). While CD has become a global disease, its etiology and pathogenesis remain unknown. Genetic susceptibility, dysregulated immune systems, and exposome-triggering factors such as microbial and dietary factors might contribute to CD development (5-7). In recent years, researchers have focused more on the role of dietary and nutritional factors in CD pathogenesis and intervention.

Exclusive enteral nutrition (EEN) is a nutritional treatment with minimal adverse effects. Studies have compared the effect of EEN to that of steroids, which are commonly used as first-line induction therapy for active CD but have several adverse side effects, including Cushing syndrome, acne, infection, and growth delay. Steroids are usually administered orally with a dosage of prednisolone at 40–60 mg/day and, subsequently, tapered by 5 mg/week to a daily dose of 20 mg, followed by a 2.5–5.0 mg weekly tapering schedule with no usage for more than 3 months (1). A meta-analysis including five randomized clinical trials that compared the effect of EEN with corticosteroids suggested that EEN was as efficacious as corticosteroids (8). Studies performed at our center have demonstrated that EEN is equally effective as steroids and biologics for inducing early clinical remission in adult patients with CD, particularly in complicated cases, such as those with intestinal fistulas, abdominal abscesses, inflammatory intestinal strictures, or treatment resistance (9,10). Specifically, EEN promotes mucosal healing (MH) and fistula closure and reduces abdominal abscess size. In the recent CD management guidelines, EEN is recommended as a first-line intervention for adolescent patients and an alternative to steroids and biological drugs for inducing disease remission in adults

(11,12). Based on these findings, dietary and nutritional factors have been increasingly recognized as determining factors in CD onset. As such, EEN-mimicking dietary therapies, such as CD-treat or the CD-exclusion diet, have been used as CD interventions. However, these diet interventions are still not comparable to EEN and cannot replace EEN (13,14).

While EEN effectively manages CD, its specific molecular mechanism remains undefined. Genetic predisposition and gene-environment interactions, as well as epigenetic modifications, such as DNA-methylation and non-coding RNAs, might be involved in CD pathogenesis (15). Since diet and nutritional components can both influence the intestinal microflora and regulate gene expression directly or indirectly via epigenetic mechanisms (16,17), analysis of nutritional component alterations after EEN intervention could help clarify its mechanism. Previous studies have reported that EEN potentially inhibits inflammation through influencing the intestinal bacterial milieu and body metabolites (18). Diederen *et al* collected stool samples from a pediatric population consisting of 43 patients with CD and 15 healthy controls (HCs). They reported that EEN decreased the diversity of microbiota, reduced amino acids, and decreased the levels of trimethylamine and cadaverine towards the control levels. Patients with CD had a reduced microbial metabolism of bile acids that was partially normalized during EEN. They further performed *in vivo* and *in vitro* experiments which confirmed that trimethylamine, cadaverine, bile salts, and amino acids could play a role in the mechanism by which EEN induces remission (19). Lipids are essential nutritional substances for life activities, as they are crucial for signal transduction, cell membrane composition, and energy storage (20). Significant changes in lipid metabolism are associated with CD; lipid metabolism and signaling are thought to play important roles in inflammation, with significant implications for IBD (21-24). Previous studies mainly focused on the clinical effect of EEN or its effect on the microbial changes in CD. Studies that investigated lipidomics in CD mainly focused on the role of the lipids in the etiology and pathogenesis of CD or its diagnostic value for the differential diagnosis of CD. The analysis of the changes in the lipid profile after EEN treatment in CD is lacking and has not been systematically investigated before.

Therefore, we conducted a comprehensive lipidomic analysis of 869 plasma lipid species using ultra-high performance liquid chromatography coupled with tandem

Table 1 Demographic characteristics of patients enrolled in the study

Characteristics	Crohn's disease (n=11)
Gender	
Male	6
Female	5
Age (years), mean ± SD	25.36±4.84
Family history, n (%)	
No	11 (100.0)
Behavior, n (%)	
B1	5 (45.5)
B2	1 (9.1)
B3	5 (45.5)
Location, n (%)	
L1	0
L2	0
L3	9 (81.8)
L4	2 (18.2)
Intestinal surgery, n (%)	
Yes	1 (9.1)
No	10 (90.9)
Perianal disease, n (%)	
Yes	9 (81.8)
Intestinal fistula/abscess, n (%)	
Yes	5 (45.5)
No	6 (54.5)
Medication history, n (%)	
Steroids	0
Mesalazine	3 (27.3)
Thiopurine	4 (36.4)
Cyclosporine	0
Anti-TNF-α agent	4 (36.4)
Methotrexate	0
Thalidomide	0

B1, non-stenotic and non-penetrating behavior; B2, stenotic behavior; B3, penetrating behavior; L1, terminal ileum; L2, colon; L3, ileum colon; L4, upper gastrointestinal tract; TNF, tumor necrosis factor; SD, standard deviation.

mass spectrometry (UHPLC-MS/MS) to determine whether plasma lipid profile changes occur during EEN intervention and their relationship with EEN in CD. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4225/rc).

Methods

Participants

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (approval No. 2022ZSLYEC-019). All participants provided informed consent before they were enrolled in the study.

A total of 11 adult patients with active CD and 17 age- and sex-matched HCs were recruited from March 2020 to December 2020. The inclusion criteria were age ≥18 years; a CD diagnosis using international criteria (1); active luminal disease confirmed with symptoms on endoscopy and computed tomography enterography (CTE)/ magnetic resonance enteroclysis (MRE); and 12-week enteral nutrition formula treatment without any other food intake. The exclusion criteria were a history of hyperlipidemia or antibiotic treatment within 2 weeks; concurrent antibiotic or anti-inflammatory agent treatment (such as steroids, thiopurines, methotrexate, and thalidomide); and patients who had ever been diagnosed with cancer or immune system diseases. Patients received enteral nutrition formulas such as Elental (Ajinomoto Pharma, Tokyo, Japan), Enteral Nutritional Suspension (SP) (NUTRICIA, New Zealand), or Enteral Nutritional Emulsion (TP) (Fresenius Kabi Deutschland GmbH, Germany), all of which are commonly used enteral nutrition formulas for the treatment of CD in China. A sufficient amount of individual daily energy supplementation was calculated for each patient according to previous studies (25,26), and the patients were followed up every 4 weeks at our center.

Data on the participants' demographics, including family history, disease location and behavior, intestinal surgery, perianal disease, intestinal fistula/abscess, and medication history, are presented in *Table 1*. The clinical classifications of CD were assessed using the Montreal Classification (27). Endoscopy was performed at baseline and 12 weeks after

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the EEN treatment. The simple endoscopic score for CD (SES-CD) was used to evaluate MH (indicated by a score <2) (28). Disease activity was assessed at baseline and week 12 using the Crohn's Disease Activity Index (CDAI). A CDAI score <150 was considered clinical remission (29). Serum inflammatory indices, such as high-sensitivity C-reactive protein (hs-CRP), platelets (PLTs), and the erythrocyte sedimentation rate (ESR), as well as nutrition-associated markers, such as body mass index (BMI), albumin (ALB), hemoglobin (Hb), and the European Nutritional Risk Screening 2002 (NRS 2002) score, were examined at baseline and week 12.

Lipidomic analysis

Sample preparation and lipid extraction

After overnight fasting, blood samples were obtained from HCs and patients with CD before EEN and 12 weeks after EEN. The plasma fraction was obtained by centrifuging the samples and was kept at –80 ℃ for further use. Lipids were extracted using the methyl-tert-butyl ether (MTBE) method.

Lipid analysis method using liquid chromatography (LC)-MS/MS

The charged surface hybrid (CSH) C18 column (1.7 μm, 2.1 mm, 100 mm; Waters, Milford, Massachusetts, USA) was chosen for the LC separation utilizing reverse phase chromatography. Then, 3 μL of the sample was injected after the lipid extracts were redissolved in 200 L of 90% isopropanol/acetonitrile and centrifuged at 14,000 g for 15 min. Acetonitrile-water (6:4 v/v), which also contained 0.1 mM ammonium formate and 0.1% formic acid, served as solvent A, while acetonitrile-isopropanol (1:9 v/v) with 0.1% formic acid and 0.1 mM ammonium formate served as solvent B. The initial mobile phase was 30% solvent B at a flow rate of 300 μL/min. After equilibrating at 5% solvent B for 10 min, it was linearly raised to 100% solvent B in 23 min.

Q-Exactive Plus (Thermo Fisher Scientific, USA) was used to acquire mass spectra in both positive and negative modes. The electron spray ionization (ESI) parameters were optimized and preset for all measurements as follows: source temperature, 300 ℃; capillary temperature, 350 ℃; ion spray voltage, 3,000 V; S-Lens radio frequency level, 50%; scanning range, 200–1,800 m/z.

LipidSearch-based identification

Based on the MS/MS calculations, LipidSearch software (Thermo Fisher Scientific, USA) was utilized to identify the lipid species. The mass tolerance for both the precursor and the fragment was set to 5 ppm.

Statistical analysis

The quantitative data are expressed as the mean ± standard deviation. Paired t-tests and Chi-squared/Fisher's exact tests were used to compare the continuous and categorical variables before and after EEN, respectively. The correlation between clinical and lipid parameters was analyzed using Pearson and Spearman correlations. $P<0.05$ was considered statistically significant. All the measurements were analyzed using SAS (version 9.4; SAS Institute, Cary, NC, USA).

The LipidSearch-extracted data were analyzed with univariate, multivariate, hierarchical cluster, and correlation analyses. The univariate statistical analysis included a student's t-test, nonparametric test, and fold change (FC) analysis. FC >1.5 or <0.67 and $P<0.05$ indicated differential lipid molecules in the univariate analysis. After data preprocessing with Pareto-scaling, a multidimensional statistical analysis was performed using SIMCA-P 14.1 (Umetrics, Umea, Sweden). An orthogonal partial least squares discriminant analysis (OPLS-DA) was used to determine the global metabolic changes between groups. The variable importance in the projection (VIP) was calculated for the OPLS-DA model. VIP >1 and $P<0.05$ indicated differential lipid molecules.

The significantly altered functional pathways were visualized with an ingenuity pathway analysis (IPA) using MetaboAnalyst 4.0 and MetScape, which are freely available online tools.

Results

Participants' demographics

From March 2020 to December 2020, 13 adult patients with active CD accepted the EEN intervention, but two patients withdrew due to intolerance of the treatment. Finally, 11 patients with CD and 17 age- and sex-matched HCs (5 women, 12 men; mean age, 27.35±4.35 years) were recruited. Their detailed demographic data are summarized in *Table 1*.
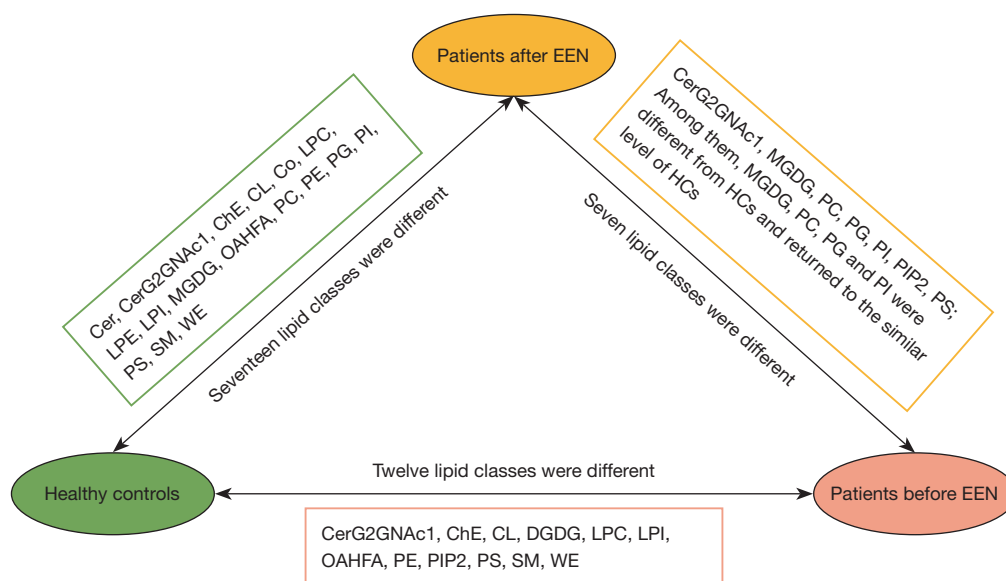
Figure 1 Different lipid classes among healthy controls and patients before and after EEN. Cer, ceramide; CerG2GNAc1, simple glucosylceramide series; ChE, cholesterol ester; CL, cardiolipins; Co, coenzyme; DGDG, digalactosyl diacylglycerols; EEN, exclusive enteral nutrition; HCs, healthy controls; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MGDG, monogalactosyldiacylglycerol; OAHFA, (O-acyl)-1-hydroxy fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP2, phosphatidylinositol diphosphate; PS, phosphatidylserine; SM, sphingomyelin; WE, wax esters.

### Changes in clinical index, clinical remission, and MH after 12 weeks of EEN

Among the 11 patients with active CD, 9 (81.8%) achieved remission and demonstrated a significant decrease in the CDAI from 291.09±78.57 before the treatment to 118.09±79.11 after the treatment (P<0.001; Table S1). One patient with a clinical response did not achieve remission; another with no response to EEN treatment received another therapy. Ten patients had an SES-CD score >2 before the treatment, and 4 (40.0%) achieved MH after the treatment (SES-CD score 6.45±2.81 vs. 3.18±2.22, P=0.013; Table S1). After 12 weeks of EEN, hs-CRP (pre-treatment vs. post-treatment: 64.94±38.38 vs. 6.54±16.26 mg/L; P=0.001), ESR (62.64±28.43 vs. 17.55±4.33 mm/h; P<0.001), and PLT [(463.00±136.88)×10^9/L vs. (298.36±107.75)×10^9/L; P=0.001] decreased significantly. The average BMI (17.05±2.02 vs. 18.64±2.36 kg/m^2; P=0.005) significantly improved, and the NRS 2002 score significantly decreased (3.91±1.14 vs. 2.55±1.29; P<0.001). ALB and Hb also improved (Table S1). These significant changes suggested a less active disease and an improved nutrition status in patients after the EEN intervention.

Plasma lipid profiles at the lipid class level

Lipid class alterations in HCs and patients before and after EEN

A total of 869 lipid species were measured using UHPLC-MS/MS and grouped into 28 classes (Figure S1). Before treatment, 17 lipid classes differed between the CD and HC groups. After the treatment, 12 classes showed differences between the two groups (P<0.05, Figure 1). The plasma levels of seven lipid classes in patients with CD changed significantly after EEN treatment, with increases in simple glucosylceramide series (CerG2GNAc1), monogalactosyldiacylglycerol (MGDG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) and decreases in phosphatidylglycerol (PG) and phosphatidylinositol diphosphate (PIP2). Notably, MGDG, PI, and PC levels were lower, and the PG level was higher in CD patients before EEN treatment than HCs (P<0.05, Figure 2). These levels normalized to HC levels after EEN treatment (P>0.05).

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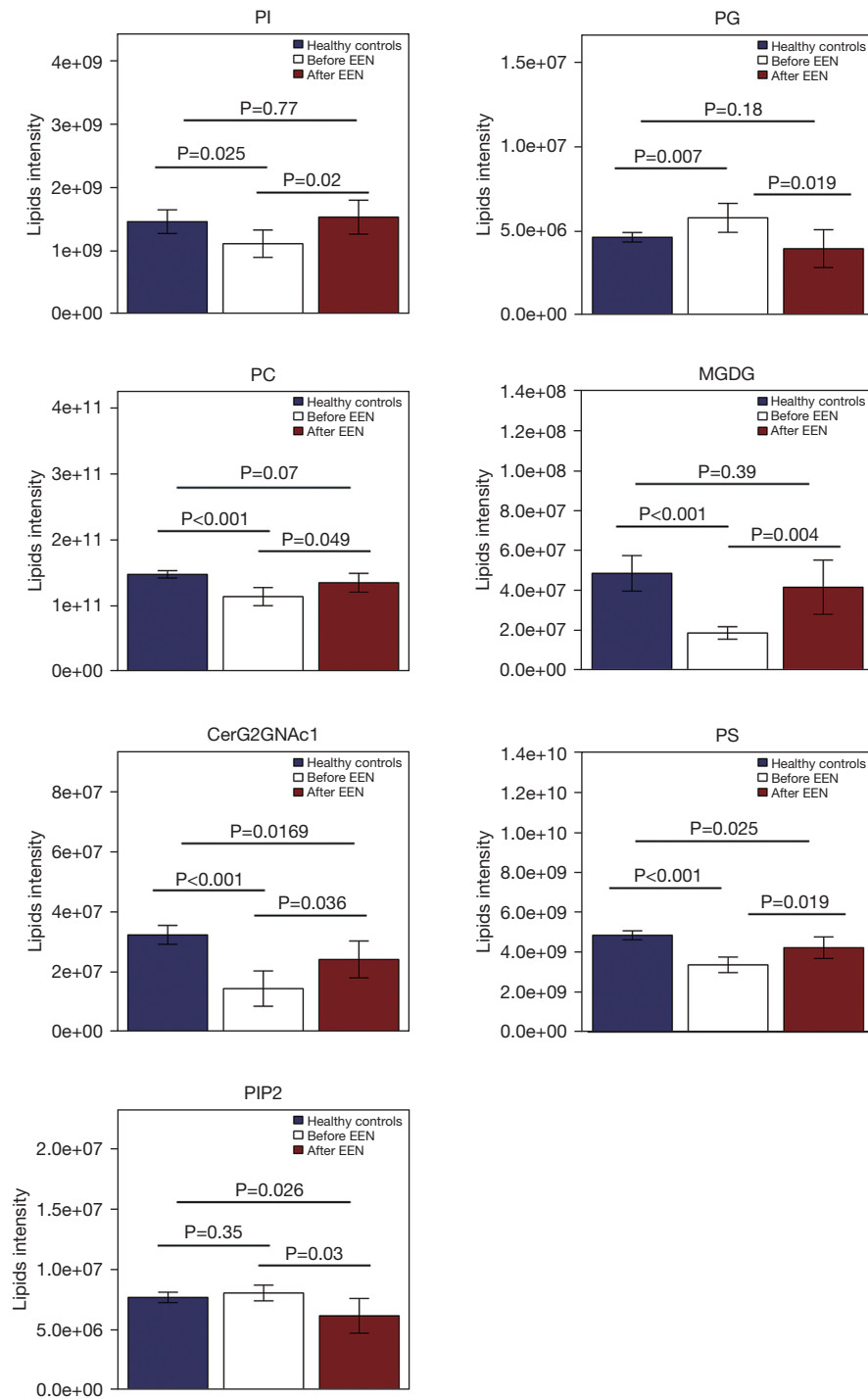
Figure 2 The plasma level of seven lipid classes altered in patients after EEN. Four of them were also different from HCs before treatment (P<0.05) and they returned to similar levels as HCs after EEN. CerG2GNAc1, simple glucosylceramide series; EEN, exclusive enteral nutrition; HCs, healthy controls; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP2, phosphatidylinositol diphosphate; PS, phosphatidylserine.

Table 2 Association of the lipid classes with disease activity and inflammatory markers in CD patients

Disease activity and inflammatory markers	Lipid classes						
	PI	MGDG	CerG2GNAc1	PC	PG	PS	PIP2
CDAI	−0.575**	−0.521*	−0.425*	−0.472*	0.476*	−0.574**	0.442*
P value	0.005	0.013	0.048	0.026	0.025	0.005	0.039
PLT	−0.319	−0.315	−0.478*	−0.420	0.477*	−0.478*	0.474*
P value	0.148	0.153	0.025	0.051	0.025	0.025	0.026
hs-CRP	−0.405	−0.481*	−0.468*	−0.215	0.591**	−0.318	0.232
P value	0.062	0.023	0.028	0.337	0.004	0.149	0.300
ESR	−0.513*	−0.465*	−0.584**	−0.451*	0.342	−0.488*	0.352
P value	0.015	0.029	0.004	0.035	0.119	0.021	0.108
SES-CD	−0.287	−0.331	−0.442*	−0.288	0.208	−0.480*	0.087
P value	0.195	0.132	0.039	0.194	0.353	0.024	0.701

*, P<0.05; **, P<0.01. CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; CerG2GNAc1, simple glucosylceramide series; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP2, phosphatidylinositol diphosphate; PLT, platelet; PS, phosphatidylserine; SES-CD, simple endoscopic score for Crohn's disease.

Associations between the lipid class levels and the clinical indices

Correlations between the lipid classes and the disease activity/inflammatory markers (CDAI, PLT, hs-CRP, ESR, and SES-CD) in CD patients were analyzed. While PI, MGDG, CerG2GNAc1, PC, and PS levels negatively correlated with the CDAI, PG levels positively correlated with the CDAI. PG and PS levels negatively correlated with PLT, while CerG2GNAc1 levels positively correlated with PLT. MGDG and CerG2GNAc1 levels negatively correlated with hs-CRP, while PG levels positively correlated with hs-CRP. PI, MGDG, CerG2GNAc1, PC, and PS levels were negatively associated with ESR. CerG2GNAc1 and PS levels negatively correlated with SES-CD (*Table 2*).

*Plasma lipid profiles at the lipid species level*

Three OPLS-DA models were used for the three pair-wise analyses of the lipid groups among CD patients and HCs pre- and post-treatment. All models displayed a clear separation among the different groups and passed the permutation test (*Figure 3*). Samples from each group were significantly separated, indicating that the lipid metabolism changed significantly due to the disease status itself and the EEN treatment. These models showed good reliability.

The Q2 of the OPLS-DA for CD patients pre- *vs.* post-treatment was 0.272, which could be partially explained by obvious differences in the disease status of each patient.

Species-level alterations in plasma lipids

The different lipid species (VIP >1.0, P<0.05) were selected based on the OPLS-DA model. Before EEN treatment, 80 lipid species significantly differed between CD patients and HCs (table available at https://cdn.amegroups.cn/static/public/atm-22-4225-01.pdf). After EEN treatment, 103 lipid species were significantly different between CD patients and HCs (table available at https://cdn.amegroups.cn/static/public/atm-22-4225-02.pdf). Significant changes in 38 lipid species, primarily belonging to the PC, triglyceride (TG), LysoPC (LPC), and PI classes, were observed between CD patients pre- *vs.* post-treatment (*Table 3*), indicating that EEN significantly altered the lipid metabolism.

To show the relationship between the different samples and the differences in the lipid expression patterns in the samples, we used the expression of qualitatively significant differential lipids, with VIP >1 and P<0.05, to conduct hierarchical clustering for each sample group. As shown in the heatmap (*Figure 4A*), the plasma lipid species before and after the EEN intervention can be divided into two large groups, which may play an important role in the

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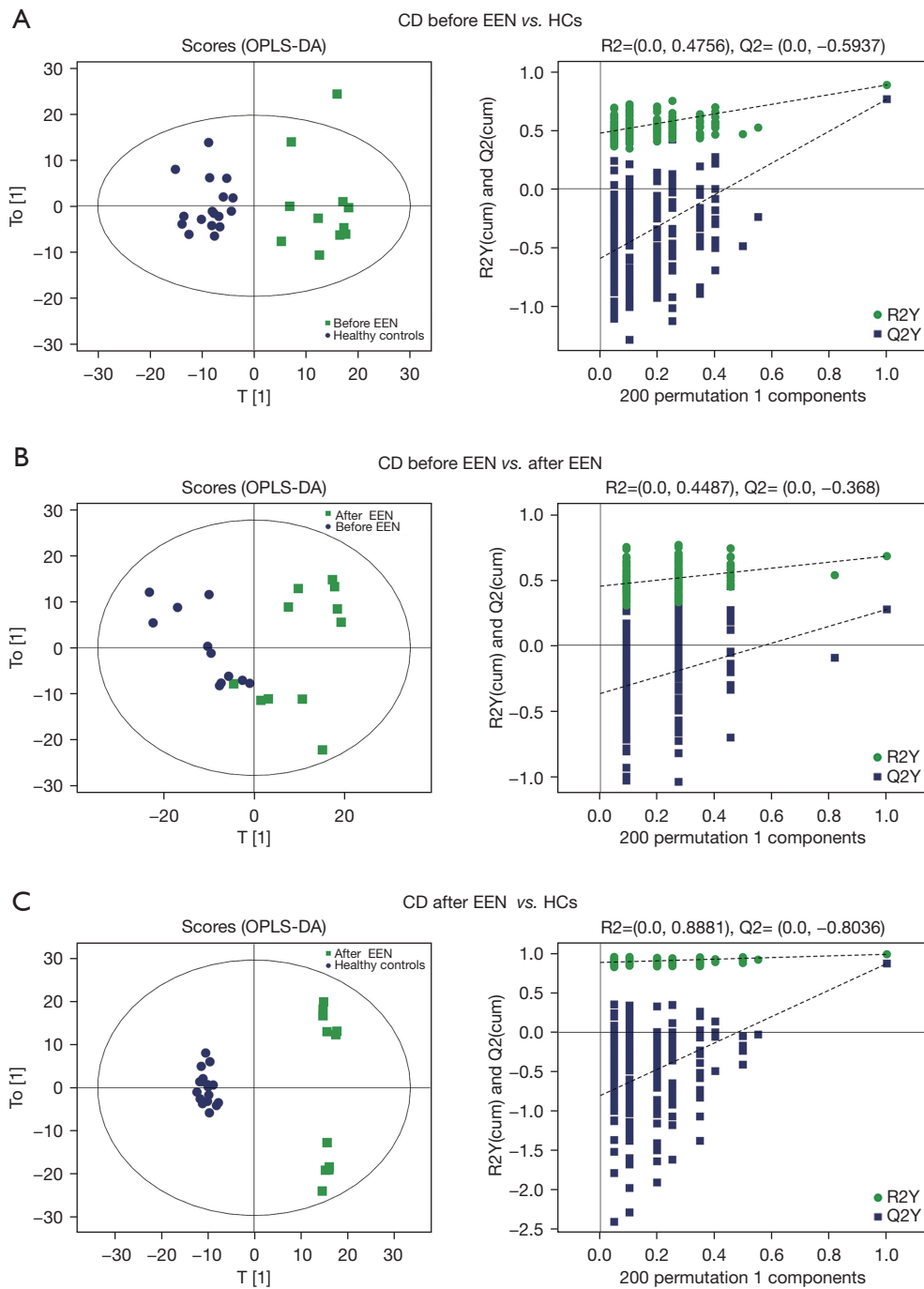
Figure 3 Multivariate data analysis of plasma lipid species in patients and HCs. (A) OPLS-DA score plot and permutation test for CD before EEN and HCs. (B) OPLS-DA score plot and permutation test for CD before and after EEN. (C) OPLS-DA score plot and permutation test for CD after EEN and HCs. CD, Crohn's disease; EEN, exclusive enteral nutrition; HCs, healthy controls; OPLS-DA, orthogonal partial least squares discriminant analysis; To [1], the first orthogonal principal component; T [1], the first predicted principal component; R2Y, the parameter for evaluating the quality of the OPLS-DA model. R2Y describes the percentage of Y matrix information that can be explained by the model; Q2Y, the parameter for evaluating the predictive ability of the model; cum, cumulative.

Table 3 Differences in lipid species in patients with CD before and after EEN

Lipid ion	Class	Ion formula	Fold change	P value	VIP
ChE(20:4)+NH4	ChE	C47 H80 O2 N1	0.62	0.016	1.64
PC(32:1)+H	PC	C40 H79 O8 N1 P1	4.41	0.006	4.64
PC(33:1)+H	PC	C41 H81 O8 N1 P1	3.94	0.019	1.33
PC(34:3)+H	PC	C42 H79 O8 N1 P1	4.70	0.034	1.32
PC(34:1)+H	PC	C42 H83 O8 N1 P1	1.49	0.019	5.35
PC(36:4)+H	PC	C44 H81 O8 N1 P1	2.83	0.006	1.57
PC(36:3)+H	PC	C44 H83 O8 N1 P1	6.67	0.007	5.32
PC(36:1)+H	PC	C44 H87 O8 N1 P1	1.96	0.021	3.73
LPC(16:0)+H	LPC	C24 H51 O7 N1 P1	1.50	0.024	1.06
PC(38:7)+H	PC	C46 H79 O8 N1 P1	0.83	0.014	1.15
PC(16:0/22:6)+H	PC	C46 H81 O8 N1 P1	4.31	0.007	1.19
PC(38:3)+H	PC	C46 H87 O8 N1 P1	6.17	0.013	3.23
TG(16:0/14:0/18:2)+NH4	TG	C51 H98 O6 N1	4.89	0.021	3.30
TG(16:0/16:0/16:1)+NH4	TG	C51 H100 O6 N1	5.16	0.031	3.78
TG(16:0/16:0/16:0)+NH4	TG	C51 H102 O6 N1	3.46	0.023	1.77
PC(40:6)+H	PC	C48 H85 O8 N1 P1	5.54	0.010	1.27
TG(16:0/16:1/18:1)+NH4	TG	C53 H102 O6 N1	2.47	0.034	5.82
TG(16:0/16:0/18:1)+NH4	TG	C53 H104 O6 N1	2.34	0.021	4.30
TG(18:0/16:0/16:0)+NH4	TG	C53 H106 O6 N1	3.56	0.031	1.63
TG(16:0/17:1/18:1)+NH4	TG	C54 H104 O6 N1	2.40	0.045	1.38
LPC(16:1)+HCOO	LPC	C25 H49 O9 N1 P1	3.33	0.013	1.54
LPC(16:0)+HCOO	LPC	C25 H51 O9 N1 P1	1.42	0.030	1.78
PE(16:1/18:1)-H	PE	C39 H73 O8 N1 P1	2.01	0.019	1.72
PE(16:0/20:4)-H	PE	C41 H73 O8 N1 P1	2.27	0.017	1.89
PE(18:1/18:2)-H	PE	C41 H75 O8 N1 P1	2.10	0.036	1.22
PC(14:0/18:2)+HCOO	PC	C41 H77 O10 N1 P1	2.39	0.022	1.00
PC(16:0/16:1)+HCOO	PC	C41 H79 O10 N1 P1	4.75	0.005	4.86
PC(17:1/16:0)+HCOO	PC	C42 H81 O10 N1 P1	3.78	0.021	1.45
PC(16:0/18:1)+HCOO	PC	C43 H83 O10 N1 P1	1.60	0.017	7.12
PI(16:0/16:1)-H	PI	C41 H76 O13 N0 P1	9.34	0.005	1.89
PC(18:2/18:2)+HCOO	PC	C45 H81 O10 N1 P1	2.80	0.005	1.62
PS(39:2)-H	PS	C45 H83 O10 N1 P1	6.07	0.008	6.08
PC(18:0/18:1)+HCOO	PC	C45 H87 O10 N1 P1	1.92	0.022	4.22
PI(16:0/18:1)-H	PI	C43 H80 O13 N0 P1	4.07	0.005	2.70
PC(18:0/20:2)+HCOO	PC	C47 H89 O10 N1 P1	1.52	0.016	1.08
PI(16:0/20:3)-H	PI	C45 H80 O13 N0 P1	10.84	0.008	1.32
MGDG(43:10)-H	MGDG	C52 H79 O10	2.23	0.004	1.05
PI(18:0/20:3)-H	PI	C47 H84 O13 N0 P1	10.03	0.033	2.62

ChE, cholesterol ester; LPC, lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triglyceride; VIP, variable importance in the projection; CD, Crohn's disease; EEN, exclusive enteral nutrition.

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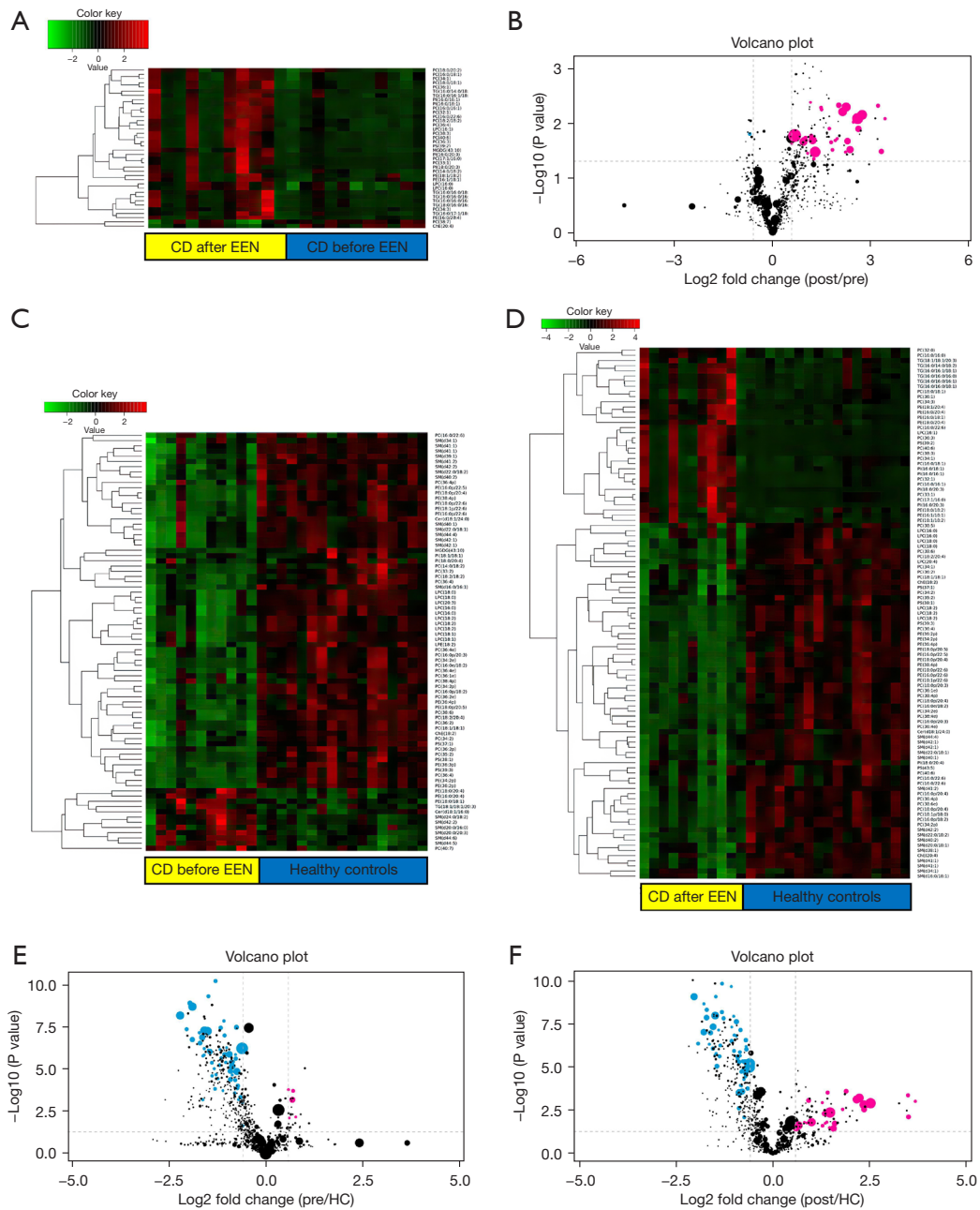
Figure 4 Heatmap and volcano plots showing significantly different plasma lipid species among different groups. (A) The heatmap of the 38 identified lipid species with significantly different plasma levels between CD patients before and after EEN. (B) The volcano plot of the different lipid species in CD patients before and after EEN. (C,D) The heatmap of significantly different plasma lipid species between healthy controls and CD patients before and after EEN, respectively. (E,F) The volcano plot of significantly different plasma lipid species between healthy controls and CD patients before and after EEN, respectively. In the volcano plot, red points (FC >1.5, P<0.05 and VIP >1) and blue points (FC <0.67, P<0.05 and VIP >1) represent the different lipids. In the heatmap, the increased or decreased levels of the metabolites (shown in rows) in different plasma samples (shown as columns) are indicated by red or green, respectively. Cer, ceramide; CD, Crohn's disease; ChE, cholesterol ester; EEN, exclusive enteral nutrition; FC, fold change; HC, healthy control; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; post, after EEN; pre, before EEN; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride; VIP, variable importance in the projection.
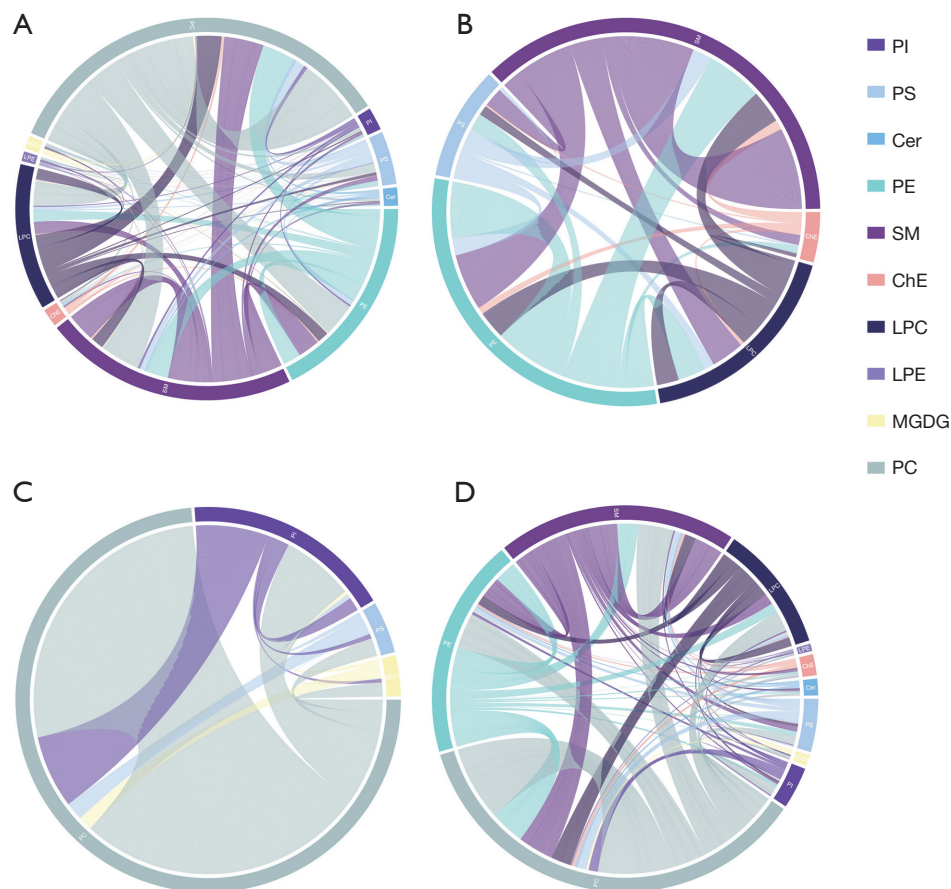
Figure 5 The circularized diagrams of the interrelationships of the significantly altered lipid molecules. The circularized diagrams displaying the significant interrelationships (Spearman, P<0.05) of the lipid classes and species with significant alterations. The different colors in the figure represent different lipid classes and molecules. Colored lines in the diagrams indicate the correlation of lipid species within/between classes. (A) The interrelationships among different expressed lipid molecules between HCs and patients before the EEN intervention; (B) the interrelationships among different expressed lipid molecules between HCs and patients after the EEN intervention; (C) the interrelationships among different expressed lipid molecules between patients before and after the EEN intervention; (D) the interrelationships among all the differentially expressed lipids that are identified in this study. Cer, ceramide; ChE, cholesterol ester; EEN, exclusive enteral nutrition; HCs, healthy controls; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

EEN mechanism and help distinguish its efficacy. The volcano plot analysis, based on VIP, P, and FC values, showed different lipid species in CD patients at pre- *vs.* post-intervention (*Figure 4B*). The hierarchical clustering analysis and volcano plots for CD patients before and after the EEN intervention compared to HCs also suggested lipid species among the different groups were altered (*Figure 4C-4F*). Correlation analysis was performed to further understand the interrelationships of the significantly altered lipid molecules, and the results suggested significant interrelationships among them (*Figure 5*).

Correlations between the lipid species and clinical indices

We analyzed the correlations between the lipid species and the disease activity/inflammatory markers in CD patients. As with the lipid classes, many lipid species negatively or positively correlated with the CDAI, hs-CRP, ESR, and
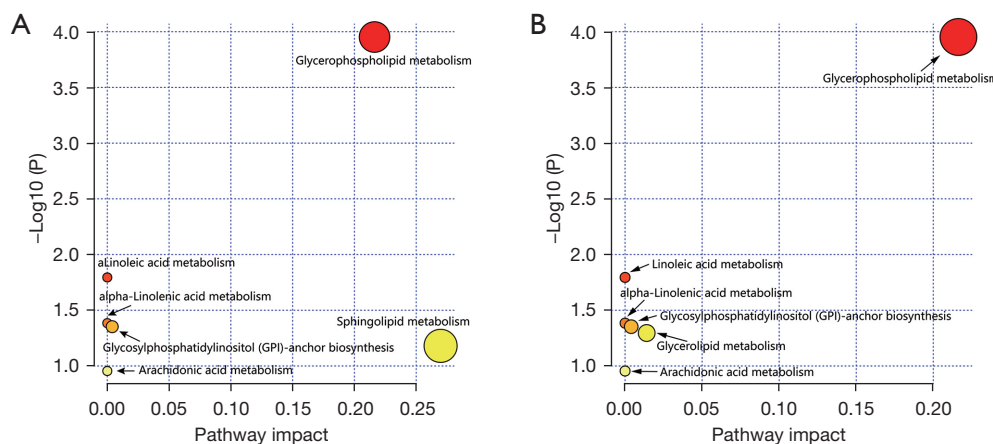
Figure 6 IPA based on different lipid metabolites among different groups. (A) IPA between HCs and CD patients before EEN; (B) IPA between CD patients before and after EEN. The P value (y-axis) and the pathway impact (x-axis) was represented by the color and the size of the circle, respectively. CD, Crohn's disease; EEN, exclusive enteral nutrition; HCs, healthy controls; IPA, ingenuity pathway analysis.

SES-CD (table available at https://cdn.amegroups.cn/static/public/atm-22-4225-03.pdf), further confirming that they may be involved in the EEN mechanism.

Altered metabolic pathways

IPA was performed to understand the function of the altered lipid species. Compared to HCs, the top five dysregulated lipid pathways in pre-intervention CD patients correlated with the metabolism of glycerophospholipids, sphingolipids, linolenic acid, and arachidonic acid (AA), as well as the glycosylphosphatidylinositol (GPI) anchor biosynthesis (*Figure 6A*). The functional pathways potentially related to EEN treatment, including the metabolism of glycerophospholipids, linolenic acid, glycerolipids, and AA, as well as the biosynthesis of the GPI-anchor, were also analyzed (*Figure 6B*). Furthermore, the network of the significantly altered metabolomic data related to the differential lipid species was also summarized by MetScape analysis, which suggested that the metabolism of AA, glycerophospholipids, linoleate, and PI phosphate was significantly altered in patients receiving EEN treatment (Figure S2).

Discussion

This study focused extensively on plasma lipid profiling of patients with CD who received EEN treatment. Significant alterations in the plasma lipids of CD patients were observed after EEN intervention. Additionally,

the levels of lipid classes and species in these patients significantly correlated with the CDAI, SES-CD, and various inflammatory biomarkers (hs-CRP, PLT, and ESR). A pathway analysis further revealed that the metabolism of AA, glycerophospholipids, linoleate, and PI phosphate was altered in CD patients, suggesting an association between these pathways and EEN.

Previous studies have shown significant alterations in the lipid profiles of patients with CD. Fan *et al.* (30) reported changes in 33 lipid species (mostly ether lipids, such as alkylphospholipids and plasmalogens) in CD patients compared to controls. Scoville *et al.* revealed that 162 serum lipid species were significantly altered in CD patients compared to controls. The levels of the long chain, polyunsaturated, branched chain, and monohydroxy fatty acids were consistently reduced in these patients. Additionally, the levels of conjugated and unconjugated primary and secondary bile acids were elevated in CD patients compared to controls (31). In a study of plasma lipid profiles of Italian IBD patients, Murgia *et al.* showed that levels of LPC 18:2, PC 18:0/18:1, PC 18:1/18:1, PC 18:2/18:2, and PC 18:3/20:4 were lower in CD patients than controls, while levels of PC 16:0/22:6, PC 18:0/22:6, PC 18:3/20:3, sphingomyelin (SM) 16:1/20:1, TG 18:1/18:2/20:4, and TG 16:0/18:1/18:2 were higher (32). A recent study also revealed that plasma levels of lysophosphatidylserine, PS, and sphingosine-1-phosphate were significantly higher in CD patients than in controls, while those of lysophosphatidylinositol (LPI) and PC were significantly lower (22). We observed

that 17 classes [ceramide (Cer), CerG2GNAc1, cholesterol ester (ChE), cardiolipins (CL), coenzyme (Co), LPC, lysophosphatidylethanolamine (LPE), LPI, MGDG, (O-acyl)-1-hydroxy fatty acid (OAHFA), PC, phosphatidylethanolamine (PE), PG, PI, PS, SM, and wax esters (WE)] and 80 lipid species were significantly different between CD patients before EEN intervention and HCs. Changes in some of these lipid classes have also been reported in previous studies (22,30-32). However, some lipids in our study were not previously investigated, and the results of different studies have been inconsistent. This may be partly due to differences in the sample collection methods and study populations (such as age and sex distributions, disease activity, dietary profiles, medications, etc.). In our study, the patients with CD were relatively young and exhibited active disease without concomitant drug use. Furthermore, the methods of lipids extraction and separation that differ among various studies may influence the results. Currently, a variety of technical methods are applied in lipid analysis, including gas chromatography-mass spectrometry (GC-MS), LC-mass spectrometry (LC-MS), and nuclear magnetic resonance. The nuclear magnetic resonance method, with low sensitivity, can only be used in tissues with high content of detectable lipids. Analysis using GC-MS is limited to the samples that can be vaporized and ionized at approximately 300 ℃ or lower. In comparison to GC-MS, LC-MS is suitable for analyzing polar and non-polar compounds. UHPLC is composed of a fixed loaded column with particle size less than 2 μm, high pressure solvent output adapted to its speed and performance, fast automatic prototype, detector, data acquisition control system, and low dead volume chromatography system that can withstand higher pressure. The UHPLC-MS/MS has higher sensitivity, separation, and analysis speed than does the conventional LC-MS. Thus, UHPLC-MS/MS represents a powerful tool for lipidomic studies and has been widely used in this field (33,34). Accordingly, we choose the UHPLC-MS/MS method to accurately analyze the plasma lipids.

Potential plasma lipid alterations induced by EEN intervention in patients with CD have not been investigated. Our results suggest that seven lipid classes (CerG2GNAc1, MGDG, PG, PC, PI, PIP2, and PS) and 38 lipid species were significantly different at pre- *vs.* post-intervention in CD patients. Among the seven lipid classes, CerG2GNAc1, MGDG, PI, PS, and PC levels increased significantly after EEN intervention in CD patients, while PG and PIP2 levels decreased significantly. Additionally, some lipid classes and species significantly correlated with

hs-CRP, CDAI, SES-CD, PLT, and ESR (*Table 2* and table available at https://cdn.amegroups.cn/static/public/atm-22-4225-03.pdf), suggesting that lipids may be involved in the mechanism of EEN. For example, the negative correlations of PS with CDAI, PLT, ESR, and SES-CD suggested that PS may inhibit inflammation in CD. A recent investigation of the role of PS in experimental colitis revealed that the anti-inflammatory effect of Annexin A5 depended on PS endocytosis, suggesting externalized PS on colonic capillaries may be utilized as a novel pharmacological target for IBD (35). Another study also reported that an increased endothelial PS level might promote a hypercoagulable state, resulting in a thrombosis risk for IBD patients (36). A higher PI level significantly correlated with a lower CDAI and ESR (*Table 2*). PI was transformed into its derivatives in the body. Moreover, inositol trisphosphate (IP3) enema, a PI derivative, promoted recovery from intestinal inflammation and increased the lifespan of mice with experimental colitis by stimulating the repair of intestinal epithelial cells by regulating intestinal histone deacetylase 3 activity (37). In summary, our study showed a correlation between identified lipid classes and species and disease activity markers, which should be confirmed in future studies with larger sample sizes and validated via mechanistic investigations.

The IPA revealed that the metabolism of glycerophospholipids, AA, linoleate, and PI phosphate was significantly altered in CD patients receiving EEN. These pathways may be involved in the EEN mechanism or CD pathogenesis. Notably, lysophosphatidylcholines (LPCs) derived from glycerophospholipids were shown to be differentially expressed in the plasma samples of rats with experimental colitis. LPCs were associated with intestinal barrier function damage by increasing ileal permeability (38). Previous studies have systematically reviewed the role of AA metabolites, including prostaglandins, thromboxanes, lipoxygenases, and leukotrienes, in IBD (39,40). These metabolites showed differential expressions in the diseased intestinal mucosa in IBD patients and immunomodulatory roles in animal studies. Although increasing evidence has shown that these lipid pathways may play pivotal roles in the inflammatory process in animal and *in vitro* experiments, their influence on EEN intervention in humans remains unclear, given the complexity of the interactions among the mixture of lipids and other compounds in the human body. Furthermore, the mechanistic impact of EEN on inducing changes in lipid metabolism is still unclear. Previous studies reported that nutrients present in the enteral formulas could regulate the lipid metabolism. For instance, carbohydrates,

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such as dextrin, are abundant in the enteral nutrition formulas and can be converted into fats in the body to regulate the lipid metabolism. Omega 3 fatty acids are also present in the enteral nutrition formulas. A randomized double-blinded placebo-controlled trial revealed that the high-dose intake of omega 3 fatty acids combined with statins has a favorable role in altering the levels of serum TGs and non-high density lipoprotein cholesterol in patients with hyperlipidemia (41). Xiong *et al.* reported that a high-iron diet could regulate lipid metabolism by significantly increasing the expression of the fat hydrolysis enzyme genes in both the liver and adipose tissues in mice (42). Han *et al.* suggested that microbiota depletion in high-fat diet-fed mice changed the expression of the hepatic genes involved in cholesterol and fatty acid metabolism (43). Accordingly, the nutrients in EEN and the changes in the EEN-induced gut microbiota may regulate the lipid metabolism through affecting gene expression, endogenous transformation, or the other mechanisms. Thus, the exact mechanism of EEN on altering lipid metabolism needs further studies. Further studies are required to explore these topics.

This study had some limitations. First, the population of CD patients treated with EEN was small, partly due to the low incidence of CD. This small sample size prevented us from performing additional analyses, such as biomarker efficacy screening. Therefore, more comprehensive studies with larger sample sizes are required. Second, only correlational analyses of lipid alterations were performed, and their clinical significance needs further confirmation. Third, our results are based on bioinformatics analysis. Whether these lipid alterations were involved in the mechanism of EEN or were simply EEN outcomes also remains undetermined. Further *in vitro* and *in vivo* experimental studies are required to confirm our results and the relationship between EEN and lipids.

Conclusions

EEN induces alterations in multiple lipid classes and species, leading to improvements in clinical response. EEN primarily altered the AA, glycerophospholipids, linoleate, and PI phosphate lipid pathways. Further studies are needed to confirm our results and the precise roles of these lipids in EEN diet therapy.

Acknowledgments

Footnote

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (approval No. 2022ZSLYEC-019), and all participants provided informed consent before they were enrolled in the study.

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