

The Assessment of Plasma Fatty Acid Profiles in Newly Diagnosed Treatment-Naïve Paediatric Crohn's Disease

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Summary

Fatty acid (FA) profiles as potentially relevant components of Crohn's disease (CD) have been insufficiently analysed. We sought to explore the plasma profiles of n-3 and n-6 polyunsaturated fatty acids (PUFAs) in newly diagnosed untreated active CD. We included 26 consecutive CD pediatric patients (<19 years) and 14 healthy controls (HCs). Disease characteristics, including inflammatory markers, dietary histories, and the Pediatric Crohn's Disease Activity Index (PCDAI), were obtained. The profiles of plasma FAs in plasma lipid classes were analysed by gas chromatography with FID detection of methyl esters. The erythrocyte sedimentation rate, C-reactive protein level and fecal calprotectin level (all $p < 0.001$) were significantly higher in CD patients than in HCs. Most changes were observed in plasma phospholipids (PLs), such as a higher content of n-3 and changes in n-6 long-chain PUFAs in the CD group. The CD group had a lower ratio of n-6/n-3 PUFAs in PLs ($p < 0.001$) and triacylglycerols (TAGs) ($p < 0.01$). Correlations of the FA content in plasma PLs with disease activity scores of CD were also observed, which were positive for the sum of monounsaturated fatty acids (MUFAs) as well as oleic acid (18:1n-9) (both $p < 0.05$). The metabolism of PUFAs is significantly altered even in treatment-naïve newly diagnosed active pediatric CD, and the content of major FAs in PLs correlates with disease activity and inflammatory markers, thus probably contributing to the still unclear early disease pathogenesis.

Key words

Children • Crohn's disease • Inflammation • Polyunsaturated fatty acids • Profiling

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Introduction

The etiology of inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) remains unresolved, and approximately 25 % of patients with IBD present before the age 20 years (Sýkora *et al.* 2018).

Dietary essential fatty acids (EFAs), linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3), the principal sources of omega 3 (n-3) and omega 6 (n-6) 18-carbon (18C) polyunsaturated FAs (PUFAs), can be converted to biologically active long-chain (LC>20) PUFAs (Chilton *et al.* 2017). LC PUFAs, their metabolites and gut environmental factors profoundly influence the immune response, and mediators of FAs may have different effects on intestinal inflammation (Saika *et al.* 2019, Nagatake *et al.* 2019). n-6 LC PUFAs (e.g. arachidonic acid [ARA], 20:4n-6) promote

pro-inflammatory effects. Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), representing n-3 PUFAs, exert anti-inflammatory properties (Serhan *et al.* 2014). n-6 FAs may inhibit the inflammation-resolving effect of n-3 derivatives (Harris *et al.* 2017, Gonzales-Gil *et al.* 2016, Innes *et al.* 2018); however, the interactions are understudied. High intakes of animal fat and n-6 LC PUFA intake were linked to higher CD prevalence (Costea *et al.* 2014, Hou *et al.* 2011), and higher dietary n-3 LC PUFA intake could alleviate the risk of CD (Scoville *et al.* 2019). Many studies have been conducted to understand the influence of dietary intervention in IBD (Lewis *et al.* 2017, Nielsen *et al.* 2005, Faegan *et al.* 2008, Marion-Letellier *et al.* 2013), yet the field remains a controversial one. These studies, however, did not take into account many interacting factors, including drugs, diet or measurable FAs. Although several studies have suggested that alterations in plasma FAs might be crucial to unravelling the underlying pathology of CD (Marton *et al.* 2019, Costea *et al.* 2014), there has been no pediatric study to evaluate the profile of FAs in newly diagnosed untreated CD.

Therefore, we decided to evaluate the FA profile in the main plasma lipid classes in newly diagnosed treatment-naïve CD children. The specific aims were as follows: (1) to compare the results of those with CD with those of healthy controls (HCs) and (2) to assess possible correlations with disease activity.

Methods

Study population and design

Flow chart of the participants and exclusion criteria

We performed a prospective study, consecutively enrolling all new-onset untreated CD patients. A total of 44 pediatric CD patients under the age of 19 years visiting our tertiary referral center were screened for eligibility in this trial. After CD diagnosis had been established and eligibility criteria had been met, 26 CD patients with newly diagnosed CD were enrolled. HCs were recruited in the same period according to the following criteria: routine health check-ups, minor elective surgery or regularly scheduled visits for non-CD-related complaints. None of these HCs had any active inflammation, a history of chronic disease or previous intestinal resection capable of influencing absorption, or any signs or family history of CD. The participants

included in the study were from the same place of residence with similar demographic variables. The criteria for exclusion were as follows: (1) concomitant organic/metabolic disease, (2) history of bowel surgery, (3) previous diagnosis of IBD, (4) abnormal liver or renal function, (5) severe colitis requiring intensive medical management, and (6) the presence of a serious comorbidity. None of the study participants were taking any drugs, CD-specific therapy or regular consumption of PUFAs at the time of sampling. Basic information was recorded. Prospective data were drawn from a departmental in-house computerized standardized interactive database as reported in our recent study (Sýkora *et al.* 2018).

Nutritional variables

We used prospective 3-day dietary records with the help of an experienced nutritionist to gather reasonable data on the children's dietary pattern. All data were subsequently entered and analysed using nutritional interactive computer software (Nutripro Software, Czech Republic, <http://Nutripro.cz>), and the intakes were then downloaded for subsequent analyses. We calculated the intake of energy, total fat, SFAs, MUFAs, and PUFAs (n-3 and n-6 FAs) for each subject to examine changes in the studied groups, including the proportions of total energy intake (g, % kcal, g/1000 kcal).

Diagnosis, disease classification and disease activity

All CD diagnoses fulfilled the revised Porto criteria (Levine *et al.* 2014) and the Paris classification, including age at diagnosis (A), disease location (L), disease behavior (B) and growth delay (G) (Levine *et al.* 2011). The Pediatric Crohn's Disease Activity Index (PCDAI) characterizes disease activity (Hyams *et al.* 2005). A score of 0 to 10 corresponds to no disease activity; mild disease equals a score of 11 to 30, and a score of 31 or greater represents moderate to severe disease activity.

Markers related to inflammation or disease activity

The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured by routine laboratory assays. Fecal calprotectin (FC) was assayed before bowel preparation using a quantitative enzyme immunoassay (PhiCal test, Calpro AS).

Analysis of plasma FAs using gas chromatography

The profile of plasma FAs in plasma lipid classes

was analysed by gas chromatography (Vecka *et al.* 2002). All pediatric subjects were required to fast overnight for a minimum of 12 h prior to blood collection and separation of plasma. Briefly, the plasma was extracted according to a study by Folch *et al.* (1957), and the lipid classes were isolated by TLC, transesterified with sodium methanolate and analysed by gas chromatography with a platform consisting of GC-FOCUS with an AI 1310 automatic sampler (Thermo Fisher Scientific SpA., Milan, Italy). A fused silica capillary column (30 m×0.32 mm ID) with chemically bonded stationary phase DB-WAXETR from J&W Scientific (Folsom) was used. The oven temperature was maintained for 1 min at 80 °C, raised to 150 °C by 20 K/min, then raised to 250 °C by 3 K/min and kept for 5 min; hydrogen was used as the carrier gas. The injector and detector temperatures were set at 250 °C. Individual peaks of FA methyl esters were identified by comparing the retention times with those of authentic standards (Nu-Chek Prep, Elysian, MN, U.S.A.).

Statistical analyses

STATISTICA CZ ver. 12 (StatSoft Inc., Tulsa, USA) software was used for statistical processing of the results. A descriptive statistical analysis was performed for baseline characteristics. Non-normally distributed variables were log-transformed. For the data analysis, significant differences between groups were assessed by the Mann-Whitney *U*-test for non-transformed variables or by the *t*-test for transformed variables. Distribution differences were calculated using the chi-square test with Yates's correction where appropriate. Correlations were evaluated with the Spearman rank test. The *p*-value was adjusted for multiple comparisons using Benjamini-Hochberg correction.

Ethical issues

The present study was conducted according to Good Clinical Practice and the Declaration of Helsinki. The research was approved by the institution's ethics review board. Written informed consent was obtained from the parents/legal guardians before participation in the study.

Results

Demographics of the study population

The baseline characteristics of the two groups, including inflammatory markers, are shown in Table 1, whereas full details of the constructs of the Paris classification and disease activity according to the PCDAI are given in Table 2. All children were predominantly of Czech ethnicity (Caucasian). A total of 42 subjects, 26 CD patients and 16 HCs, were enrolled in this study. There were no significant differences between the 2 studied groups except for the highly increased values of inflammatory markers in the CD patients (all *p*<0.001).

Dietary assessment

The subjects' mean daily nutrient intake is summarized in Table 3. The median time between sampling and completion of all dietary assessments was 4.5 (range 1-12) days. No significant differences were observed in the total energy intake, total fat and FA composition of the dietary intake in the CD patients (SA, MUFA, PUFA, n-3 FA and n-6 FA) when compared with levels in HCs (*p*>0.05). Energy from fat in children's diets did not exceed 30 % in either group.

Table 1. Basic characteristics and inflammatory markers of the study population.

Parameter	Controls (n=16)	CD (n=26)
Gender (female/male)	7/9	12/14
Age (years)	15 (14-17)	15 (13-16) [#]
BMI (kg.m ⁻²)	17.9 (16.2-20.1)	17.8 (15.1-20.6)
FC (μg/g)	100 (100-101)	1638 (730-1800)***
CRP (mg/l)	1 (1-1)	15 (5-45)***
ESR 1 h (mm)	7 (3-9)	30 (24-48)***
ESR 2 h (mm)	12 (9-16)	68 (51-100)***

Values are presented as mean (25th-75th percentile) unless otherwise stated. Absolute patient numbers for given categories are presented. *** – Mann-Whitney test, *p*<0.001, [#] – age at diagnosis of CD, CRP – C-reactive protein, ESR – erythrocyte sedimentation rate, CD – Crohn's disease, BMI – body mass index; [weight (kg)]/[height(m²)], FC – fecal calprotectin.

Table 2. Demographics of Crohn's disease (CD) patients enrolled per Paris classification and PCDAI scores.

Age at diagnosis	
A1a: <10 years of age	3.8 % (1/26)
A1b: 10 to <17 years	69.2 % (18/26)
A2: 17 to <40 years	27 % (7/26)
Disease activity at onset (PCDAI)	
Quiescent <10	3.8 % (1/26)
Mild to moderate 11-30	42.3 % (11/26)
Severe >31	53.9 % (14/26)
Disease location	
L1: terminal ileum/ileocecal	19.2 % (5/26)
L1 + L4a	7.7 % (2/26)
L1 + L4b	3.8 % (1/26)
L1 + L4ab	7.7 % (2/26)
L2: colonic	23.1 % (6/26)
L2 + L4a	7.7 % (2/26)
L2 + L4b	7.7 % (2/26)
L2 + L4a/b	7.7 % (2/26)
L3: ileocolonic	57.7 % (15/26)
L3 + L4a	38.5 % (10/26)
L3 + L4b	15.4 % (4/26)
L3 + L4ab	3.8 % (1/26)
L4: isolated upper tract disease	
All upper digestive involvement	88.5 % (23/26)
L4a	61.6 % (16/26)
L4b	26.9 % (7/26)
Disease behavior	
B1 inflammatory (non-stricturing, non-penetrating)	65.4 % (17/26)
B2 stricturing	3.8 % (1/26)
B3 penetrating	23.1 % (6/26)
B2/B3 stricturing & penetrating	7.7 % (2/26)
Positive ANCA	57.7 % (15/26)
Positive ASCA	46.2 % (12/26)
Perianal disease (p)	23.1 % (6/26)
Growth delay (G1)	3.8 % (1/26)

As defined by Levine *et al.* (2011). Absolute patient numbers for given categories are presented, values also represent their corresponding percentage of the overall category. ASCA – anti-*Saccharomyces cerevisiae* antibodies, PCDAI – Pediatric Crohn's disease activity index, L4a – proximal upper involvement of the angle of Treitz, L4b – distal upper involvement of the angle of Treitz and proximal to the third distal of the ileum.

Diagnostic work-up

The Paris classification

Table 2 indicates the distribution of CD patients regarding the Paris classification and PCDAI. No patients within the groups had a history of bowel surgery.

Plasma FA profiles

Significant differences in the FA profiles are displayed in [Tables S1 and S2](#) in the Supplementary Materials. The profile of three major plasma lipid classes was analysed: phospholipids (PLs), triacylglycerols

(TAGs), and cholesteryl esters (CEs), as detailed in [Table S1](#). Compared with HCs, for PLs and TAGs, we observed higher proportions of n-3 LC PUFAs in CD

patients (both $p < 0.001$), which was caused by higher contents of DHA (22:6n-3), EPA (20:5n-3) and plasma 22:5n-3 FAs.

Table 3. Dietary intake of fat in pediatric Crohn's disease (CD) patients and controls.

Variable	CD (n=26)	Controls (n=16)
<i>Total energy/per kg of body weight</i>		
(kcal)	2037 (160.1)/41.2 (2.9)	1892.5 (173.5)/38.7 (2.4)
(kJ)	8351.7 (656.4)/170.6 (11.9)	7920.6 (726.6)/161.5 (10.4)
<i>Total fat (g)</i>	61.8 (18.8)	58.5 (16.6)
(g/1000 kcal)	30.3 (2.4)	30.9 (2.5)
(En %)	27.3 (2.0)	28.8 (2.0)
<i>Total SFA (g)</i>	25.1 (9.6)	21.1 (8.9)
(g/1000 kcal)	11.3 (3.1)	12.4 (3.4)
(En %)	11.5 (4.0)	9.9 (3.6)
<i>Total MUFA (g)</i>	13.1 (7.4)	14.8 (8.1)
(g/1000 kcal)	6.4 (3.9)	8.8 (4.2)
(En %)	5.8 (2.1)	7.1 (2.8)
<i>Total PUFA (g)</i>	6.7 (1.9)	5.1 (1.6)
(g/1000 kcal)	3.3 (1.6)	3.1 (1.5)
(En %)	2.1 (1.0)	2.4 (0.8)
<i>Total n-3 (g)</i>	0.5 (0.3)	0.3 (0.2)
<i>Total n-6 (g)</i>	1.7 (1.1)	1.4 (1.0)

All differences between groups do not reach statistical significance. Absolute patient numbers for given categories are presented. Observed variables are expressed as the mean (standard error) of average daily intake. P values were calculated by Mann-Whitney U test. CD – Crohn's disease patients, En % – as percentage of total caloric intake, SFA – Saturated Fatty Acids, MUFA Monounsaturated Fatty Acids, PUFA – Polyunsaturated Fatty Acids, NS – not significant differences ($p > 0.05$) vs. healthy controls.

Individual FAs of the PUFA n-6 family exhibited a more complicated pattern of changes. In plasma PLs, a higher abundance of arachidonic acid (AA, 20:4n-6) prevailed with a lower content of linoleic acid (LA, 18:2n-6) in the CD group. Among TAGs, the content of AA was increased in the CD group ($p < 0.01$) but did not affect the respective sum. The content of LA in the plasma CEs of CD children was substantially lower ($p < 0.01$) without changes in the sum of n-6 PUFAs, as the content of AA tended to increase in plasma CE. The changes in the content of both families of PUFAs resulted in a lower ratio of n-6 PUFAs/n-3 PUFAs in the CD group in PLs ($p < 0.001$) and TAGs ($p < 0.01$).

Major FAs (MUFAs) exhibited only moderate changes between the CD and HC groups. Oleic acid (18:1n-9) was only slightly decreased in plasma PLs in

the CD group ($p < 0.05$). In PLs and CEs, the content of cis-vaccenic acid (18:1n-7) was higher in the CD group (all $p < 0.05$).

The concordant decrease in the PL content of major saturated FAs and palmitic and stearic acids brought about a profound decrease in the saturated FA (SFA) ratio in the CD group ($p < 0.001$). For TAGs and CEs, the sum of SFAs did not change.

Plasma FA profile, disease activity and inflammatory markers

Correlations between FAs, markers of inflammation and disease activity

The correlation coefficients between inflammatory and clinical parameters and the FAs are entirely outlined in [Table S2](#). We found a positive

association between the content of MUFAs in plasma PLs and the disease activity index ($p < 0.01$) and negative correlations ($p < 0.05$) between the content of several n-6 PUFAs and DHA in the plasma TAG fraction.

Discussion

Herein, this is the first published report on the abnormalities of plasma FAs in a unique CD cohort of newly diagnosed and therapy-naïve pediatric patients with CD. We have shown that the FA profiles in pediatric CD patients compared to HCs significantly differed. We observed that PUFA metabolism, mainly linked to higher contents of n-3 and n-6 LC PUFAs in PLs and a lower ratio of n-6/n-3 PUFAs in TAGs and PLs, was significantly altered even in new-onset untreated CD. Furthermore, the content of major FAs in PLs significantly correlated with disease activity. Although the pathophysiological relationship is not clear, these data may provide new options for research on pathological processes responsible for the impaired profiles in the early stage of CD.

Many records concerning the effects of PUFAs in pediatric CD have indicated mixed conclusions (Levy *et al.* 2000, Trebble *et al.* 2003, Socha *et al.* 2005, Usami *et al.* 2003). In this context, ignoring potential confounding factors, we commend the authors of the 3 aforementioned studies for identifying the metabolism of PUFAs in children with IBDs that may, at least to some extent, be compared to ours. Our reference group exhibited lower values of n-3 LC PUFAs than the reported German population (Decsi *et al.* 1994) and Swedish data (Steel *et al.* 2006). Our conclusions are closer to values reported by Grogan *et al.* (2012) for whole plasma. Conversely, the content of n-6 LC PUFAs in our HCs was considerably higher than previously reported (Grogan *et al.* 2012), mainly due to the increased proportion of LA (18:2n-6).

As previously reported (Trebble *et al.* 2003), dietary intake in children with IBD was comparable to HC. Another study explored adults with CD and those without CD; the CD group had altered profiles and higher total energy intake, but the values of the fat intake and PUFAs did not reach statistical significance (Scoville *et al.* 2019). Our analyses share a number of similarities with Scoville *et al.*'s findings (Scoville *et al.* 2019). Thus, we cannot very likely attribute the observed changes to alterations in dietary intake; moreover, samples of untreated patients were taken while fasting,

making it unlikely that these relationships are driving the profile differences between the two groups. Additionally, the mean BMI was similar in both groups, and HCs had no signs of digestive problems resulting in lower n-3 LC PUFA absorption.

Higher contents of n-3 LC PUFAs, ALA and DHA and decreased contents of n-6 PUFAs were described in adults (Esteve-Comas *et al.* 1992), even in inactive forms (Esteve-Comas *et al.* 1993). Low levels of n-6 PUFAs with increased levels of ALA were reported in children (Socha *et al.* 2005). These results are not consistent with ours obtained in this study. The contrasts are probably related to patient selection bias (previous studies were limited by the lack of untreated subjects). A possible explanation for our findings is that less efficient metabolism/utilization of n-3 FAs into anti-inflammatory resolvins, maresins and protectins, which are synthesized from DHA and EPA (Serhan *et al.* 2014), diverts PUFA metabolism towards the n-6 biosynthetic pathways that are involved in producing and modifying pro-inflammatory mediators, supporting the concept of enhanced PUFA biosynthesis. We also observed a high content of ARA in plasma PLs, which is usually linked with high ARA dietary intake as a consequence of increased consumption of animal fat, one factor known for increasing the prevalence of CD (Costea *et al.* 2014), probably due to the provision of substrates for pro-inflammatory eicosanoids. Our result of an aberrant profile in PL also coincided with that of Geerling *et al.* (1999). In fact, higher ARA/LA levels and reductions in oleic acid were observed in inflamed colonic mucosa and fecal samples derived from CD patients, consistent with the fact that the plasma patterns reflect FAs at tissue sites of inflammation (Fernández-Banares *et al.* 1997, Jansson *et al.* 2009). Several studies have supported biochemical interactions between FAs, FA-derived mediator production and immune reactions (Serhan *et al.* 2009, Yaqoob *et al.* 2007). PUFAs correlated with pro-inflammatory cytokines and disease activity, as reported previously (Scoville *et al.* 2019). It is quite plausible that the influence of FAs *via* ARA derivatives might be of relevance in the inflammatory response through different processes that downregulate inflammatory changes and immune modulations at an early stage of the disease.

In the current study, negative correlations of n-6 LC PUFAs and EPA in TAGs with disease activity were in concordance with a higher content of these FAs in CD patients versus HCs and with the higher activity of the disease demanding more substrate (ARA and EPA,

respectively) for the production of mediators. Scoville *et al.* presented an inverse correlation with LA and n-6 LC PUFAs (Scoville *et al.* 2019) in CD, but they observed a positive correlation of EPA with disease activity. Of note, major MUFAs exhibited only moderate changes between the studied groups. We demonstrated that the content of oleic acid (18:1n-9) and the sum of MUFAs in PLs positively correlated with disease activity, which contradicted the report of Perreault *et al.* (2014). They reported only weak associations between oleic acid and inflammatory markers in young adults, probably due to the absence of inflammation in the population of healthy young subjects. Our data might be relevant to the underlying mechanisms in the context of altered metabolism/utilization.

To relate our findings to objective inflammatory markers in active CD (Daniluk *et al.* 2019), we found that the profiles correlated significantly with the inflammatory state in the 2 studied groups we enrolled. However, not all FAs from the same classes were associated with inflammatory markers in active CD. Briefly, correlations of FAs with inflammatory markers were proven for some PUFAs for pooled groups (data not presented), but our results document a relatively weak relationship within the CD group. However, the findings supported by Trebble *et al.* (2003) could be explained by considerably larger differences obtained between the HC and CD groups than within the CD group. These findings imply that individual FAs may have distinct influences on the development of CD.

Clear strengths and innovative aspects of this study are the inclusion of new-onset and therapy-naïve patients with CD, the prospective design and accurate description of the participants. We focused on untreated CD children to increase the homogeneity of the study population, reducing the risk of biased estimates and the possible confounding influences on the changes in the early decline of FAs other than disease-specific activity. Specific data on dietary records in the diet of the subjects were available; thus, the likely differences in the diet between patients and HCs were of lesser impact. Thus, the observed differences were unrelated to ongoing medication, diet and sampling and reflected the state of

active untreated disease. This study had limitations. We also cannot comment on whether there were differences between different disease locations regarding the heterogeneous disease characteristics because various disease subgroups had smaller numbers of participants. Another weakness may be the lack of data on histopathology in HCs, but FC is a surrogate marker of intestinal inflammation (Daniluk *et al.* 2019). Using a self-report method to assess dietary intake may reduce the potential validity of our data due to the risk of over- or under-reporting as well as person-specific sources of bias; nevertheless, these techniques were used previously (Jackson *et al.* 2008). Although the study determined an unequivocal association, we could not prove causality, but it is a very good indicator of the disease; therefore, our data analysis must be interpreted carefully. Nevertheless, if a significant association was found, it is important to make it clear that it was from a hypothesis-generating study; consequently, larger prospective confirmatory studies are required to clarify the strength of any association.

In conclusion, our findings implicate deteriorated utilization/metabolization of PUFAs in CD, even in new-onset treatment-naïve children. We observed significant disturbances in the FA profile in newly diagnosed untreated CD patients distinct from HCs, notably LC PUFAs. The content of major FAs in PLs strongly correlated with disease activity and the inflammatory response. Our data suggest that additional assessments of the unbalanced n-6/n-3 ratio may provide clues regarding the underlying pathogenesis, in particular CD. Finally, the specific mechanisms by which abnormalities in FA profiles, most notably PUFAs, affect the early stage and progression of CD are still unknown. Nonetheless, the current findings highlight areas for larger prospective studies for this purpose.

Conflict of Interest

There is no conflict of interest.

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