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Low unesterified:esterified eicosapentaenoic acid (EPA) plasma concentration ratio is associated with bipolar disorder episodes, and omega-3 plasma concentrations are altered by treatment

**Running head:** EPA in bipolar disorder

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## Abstract

**Objectives:** Omega (n)-3 and n-6 polyunsaturated fatty acids (PUFA) are molecular modulators of neurotransmission and inflammation. We hypothesized that plasma concentrations of n-3 PUFA would be lower and of n-6 PUFA higher in subjects with bipolar disorder (BD) compared to healthy controls (HC), and would correlate with symptom severity in subjects with BD, and that effective treatment would correlate with increased n-3 but lower n-6 PUFA levels. Additionally, we explored clinical correlations and group differences in plasma levels of saturated and monounsaturated fatty acids.

**Methods:** This observational, parallel group study compared biomarkers between HC (n = 31), and symptomatic subjects with BD (n = 27) when ill and after symptomatic recovery (follow-up). Plasma concentrations of five PUFA [linoleic acid (LA), arachidonic acid (AA), alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA)], of two saturated fatty acids (palmitic acid and stearic acid) and of two monounsaturated fatty acids (palmitoleic acid, oleic acid) were measured in esterified (E) and unesterified (UE) forms. Calculated ratios included UE:E for the five PUFA, ratios of n-3 PUFA (DHA:ALA, EPA:ALA, EPA:DHA), and the ratio of n-6:n-3 AA:EPA. Comparisons of plasma fatty acid levels and ratios between BD and HC groups were made with Student *t*-tests, between the BD group at baseline and follow-up using paired *t*-tests. Comparison of categorical variables was performed using Chi-square tests. Pearson's *r* was used for bivariate correlations with clinical variables, including depressive and manic symptoms, current panic attacks, and psychosis.

**Results:** UE EPA was lower in BD than HC, with a large effect size (Cohen's *d* = 0.86, *p* < 0.002), however, it was not statistically significant after correction for multiple comparisons. No statistically significant difference was seen in any plasma PUFA concentration between BD and HC after Bonferroni correction for 40 comparisons, at *p* < 0.001. Neither depressive severity nor mania severity was correlated significantly with any PUFA concentration. Exploratory comparison showed lower UE:E EPA in BD than HC (*p* < 0.0001). At follow-up in the BD group, UE, E DHA:ALA, and UE EPA:ALA were decreased (*p* < 0.002). Exploratory correlations of clinical variables revealed that mania severity and suicidality were positively correlated with UE:E EPA ratio, and that several plasma levels and ratios correlated with panic disorder and psychosis. Depressive severity was not correlated with any ratio. No plasma fatty acid level or ratio correlated with self-reported n-3 PUFA intake or use of medication by class.

**Conclusions:** A large effect size of reduced UE EPA, and a lower plasma UE:E concentration ratio of EPA in the symptomatic BD state may be an important factor in vulnerability to a mood state. Altered n-3 PUFA ratios could indicate changes in PUFA metabolism concurrent with symptom improvement. Our findings are consistent with preclinical and postmortem data and suggest testing interventions that increase n-3 and decrease n-6 dietary PUFA intake.

**Key words:** bipolar disorder – eicosapentaenoic acid – inflammation – omega-3

Bipolar disorder (BD) is an episodic illness affecting 1.0–4.4% of the population (1). It is a highly heritable polygenic disorder (2), which has a complex, incompletely understood etiology (3). BD type I is characterized by manic episodes of elevated mood, energy and cognition, and by major depressive episodes of lowered mood, energy and cognition. BD type II is characterized by hypomanic episodes of less severity than manic episodes, and by major depressive episodes that are often longer and more difficult to treat than BD type I (4-7). Improvements in acute and maintenance treatments are needed to prevent relapses and reduce burden of disability.

Alterations of metabolism of lipids and resultant changes in cell signaling pathways have been hypothesized to perturb neurotransmitter systems in mood disorders (8, 9). Arachidonic acid [(AA) 20:4n-6] and docosahexaenoic acid [(DHA) 22:6n-3] are polyunsaturated fatty acids (PUFA) that compose over 90% of essential fatty acids in the mammalian brain (10). AA and DHA are derived from the diet or are synthesized in the liver from their respective, nutritionally essential, shorter-chain PUFA, linoleic acid [(LA) 18:2n-6] and alpha-linolenic acid [(ALA) 18:3n-3]. AA, DHA, and their metabolites function as intracellular second messengers during neurotransmission and as modulators of neuroinflammation and other pathological processes (8, 11).

Finding common changes in neurobiological systems among effective mood stabilizing medications, proven effective in Phase III trials, is a potential window into understanding etiological and treatment mechanisms in BD. One suggested common mechanism of action of mood stabilizers, derived from pre-clinical studies, is downregulation of brain AA metabolism (12-14). Epidemiological and clinical data support the hypothesis that altered PUFA metabolism is present in BD, including reduced n-3 PUFA metabolism (15-21).

We hypothesized that plasma concentrations of n-3 PUFA would be lower and plasma concentrations of n-6 PUFA would be higher in subjects with BD who experience manic or depressive symptoms than in healthy controls (HC), and that n-3 PUFA would increase and n-6 PUFA decrease after naturalistic treatment. We also hypothesized that lower n-3 and higher n-6 PUFA concentrations would be associated with manic and depressive symptoms, regardless of medication status. To test these hypotheses, we studied subjects with BD who were in a symptomatic episode while on medication.

In an exploratory analysis, we investigated associations between plasma n-3 and n-6 PUFA concentrations and clinical symptoms of panic, psychosis, and suicidality. We measured both unesterified (UE) and esterified (E) concentrations of nine fatty acids in plasma. We studied plasma concentrations because the UE form of the PUFA passes through the blood-brain barrier into brain more readily than does the E form (22, 23); thus the UE plasma concentration is the major peripheral form that represents PUFA metabolism in brain.

## Methods

### Participants and measures

Participants were recruited when presenting for care during mood episodes at the Pennsylvania Psychiatric Institute, under Institutional Review Board Protocol No. 39164EP and NIH Office of Human Subject Research Exemption #11509 (7/16/2012). Participants were screened and consented for the study. Patients with BD (i.e., bipolar disorder type I and bipolar disorder type II) and HC with no personal or family history of mood or psychotic disorder were included. Exclusion criteria included daily use of anti-inflammatory medications, active substance intoxication or withdrawal, or pregnancy. The Mini Neuropsychiatric Interview (24), a DSM-IV-TR-based structured interview, was performed, and current mood state was assessed with the Hamilton Depression Rating Scale-21 (HDRS) plus Atypical (25) and the Clinician-Administered Rating Scale for Mania (CARS-M) (26). Subjects completed rating scales including a Food Frequency Questionnaire (FFQ) for assessment of intake of n-3 PUFA (27). Demographic variables of interest included age and sex. Clinical variables of interest included depression severity (HDRS-21 + Atypical) and mania severity (CARS-M). Use of tobacco by smoking in the past month, alcohol use in the past month above recommended sex-specific limits (as recommended by the National Institute of Alcohol Abuse and Alcoholism: men, > 14 drinks/week or four

drinks per occasion, and women, > 7 drinks/week or three drinks per occasion) were recorded from subject response (28). Use of antidepressant, antipsychotic, or mood-stabilizing medications was recorded from subject response. Height and weight were measured and recorded. Clinical features of illness including presence of current panic disorder, current suicidality, and current psychosis were obtained from the relevant MINI modules. Current suicidality accounted for history of suicidal attempts, and current intent and plan. A dietary report of ALA, DHA, and EPA was calculated from self-reported intake of omega-3 rich foods recorded on the FFQ.

Subjects were followed and treated as usual. At the start of the study, subjects were taking mood stabilizers (n = 21, 78%: lithium n = 14, carbamazepine n = 3, valproic acid n = 3, and lamotrigine n = 3), antipsychotics (n = 15, 56%: aripiprazole n = 1, haloperidol n = 2, risperidone n = 6, quetiapine n = 6, perphenazine n = 1, and olanzapine n = 2), antidepressants (n = 13, 48%), and/or sedatives (n = 17, 63%). At follow-up, subjects were taking mood stabilizers (n = 9, 69%: lithium n = 4, carbamazepine n = 1, valproic acid n = 1, and lamotrigine n = 1), antipsychotics (n = 7, 54%: aripiprazole n = 2, risperidone n = 3, perphenazine n = 1, and olanzapine n = 2), antidepressants (n = 4, 31%), and/or sedatives (n = 6, 46%). After discharge from the hospital, subjects were followed each week by phone and assessed for clinical improvement. A return visit was scheduled with repeat measures when the subject was asymptomatic, or after three months had elapsed. However, due to irregular contact with some subjects, the maximum number of days for follow-up was 187 (median length of follow-up = 22 days, mean length of follow-up = 52 days).

#### Sample collection and biochemical analysis

Participants fasted for at least six hours, and blood was drawn in vacutainers containing EDTA for biomarkers in the AM. After centrifugation for 10 min at 3000 rpm, the plasma supernatant was transferred to plastic tubes and maintained at -80°C or on dry ice until processed. Samples were processed for fatty acids after thawing in a blinded fashion at the Brain Physiology and Metabolism Section, NIA.

Each plasma sample (0.5 mL) was extracted using the method described by Folch et al. (29) and Sublette (17). The plasma was mixed with a partition system of 3.0 mL chloroform:methanol (2:1) and extracted with 0.6 mL 0.1 M KCl. Organic extracts were concentrated under N<sub>2</sub> at 45°C, and then suspended in

0.5 mL chloroform. Standards and samples were applied to Silica gel 60 TLC plates and the lipids were separated using heptane:diethyl ether:acetic acid (60:40:3) (30). Plates were sprayed with 0.03% TNS (6-p-toluidine-2-naphthalene sulfonic acid) in 50 mM Tris buffer (pH 7.4) and lipid bands were visualized under UV light. Bands corresponding to UE fatty acids were scraped off and then directly methylated using 1% H<sub>2</sub>SO<sub>4</sub> in methanol (v/v) and fatty acid methyl esters (FAMES) were extracted with heptane (31). Prior to methylation, heptadecanoic acid (17:0) was added as an internal standard. For E fatty acid determinations, 50 µL plasma extract was concentrated under N<sub>2</sub> at 45°C, then directly methylated as above. FAMES were separated using a gas chromatograph (GC) (Model 6890 N; Agilent Technologies, Palo Alto, CA, USA) with a capillary column (SP 2330, 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA, USA) and a flame ionization detector (32). Plasma fatty acid concentrations (nmol/mL) were calculated by direct proportional comparison of GC peak areas with that of the added 17:0 internal standard.

#### Data and statistical analysis

SPSS version 20, IBM SPSS Statistics was used for all statistical calculations. Values of continuous variables were compared between BD and HC groups using two-sample t-tests, and categorical variables were compared using the Pearson chi-square test. Paired t-tests were used for baseline and follow-up measurements.

*Hypothesis-driven analysis.* Outcome variables included the E and UE plasma forms of five PUFA, including LA, AA, ALA, DHA, and eicosapentaenoic acid (EPA, 22:5n-3). Comparisons of (i) plasma fatty acid levels were made between the BD and HC groups using Student *t*-tests, (ii) between BD baseline and follow-up of plasma fatty acid concentrations using paired *t*-tests, and (iii) Pearson's *r* correlations between mean PUFA concentrations and mania severity, and between mean PUFA concentrations and depression severity, also were performed. Thus, 40 comparisons were investigated in a hypothesis-driven manner. An alpha = 0.05/40 = 0.001 was used as a significance level for these comparisons.

In an *exploratory analysis*, comparisons were made between the BD and HC groups, and between baseline and follow-up of plasma concentrations in the UE and E forms of the saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0), and of the monounsaturated fatty acids, palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9). Additionally, the ratios of UE to E concentrations of the five PUFA were calculated to determine the relations between unbound to bound forms; the unbound form

crosses the blood-brain barrier more easily (22, 23). Ratios of n-3 PUFA concentrations to each other (DHA to ALA, EPA to ALA and EPA to DHA) in the UE and E forms were constructed to estimate progression in the n-3 metabolism pathway. The UE and E AA:EPA ratios were calculated to estimate the n-6/n-3 ratio, an indication of the balance of the overall body PUFA cascade and their bioactive metabolites involved in neurotransmission and the resolution of inflammation (11, 33, 34). All calculated ratios were compared between the HC and BD groups, and between baseline and follow-up.

Additional exploratory analysis was used to investigate associations between demographic and clinical variables and the fatty acid concentrations and ratios described above. Pearson's *r* was used for bivariate correlations. Bivariate correlations were made between the fatty acid UE to E concentration ratios and n-3, n-3/n-6 ratios described above and the following demographic and clinical variables: age, sex, depression severity (HDRS-21 + Atypical), mania severity (CARS-M), smoking (past month), alcohol (use in the past month above recommended guidelines), current panic disorder (MINI), current suicidality (MINI), dietary report of ALA, DHA and EPA (FFQ), and use of antidepressant, antipsychotic or mood-stabilizing medications.

## Results

### Demographic and clinical description of the sample

A cohort of 27 patients with BD was recruited while in a symptomatic mood episode, and a parallel HC group was recruited for this study. Patients with BD had an average of 35 years at entry to the study and did not differ in age, gender distribution, race, or ethnicity from the HC group ( $n = 31$ ) (Table 1). The subjects with BD were less likely to be married, and less likely to be employed or a student than the HC group (Table 1).

The subjects with BD were significantly heavier than the HC subjects, with a mean body mass index (BMI) in the obese range [BD: 30.5 (6.0), HC: 24.9 (4.7);  $p = 1.7 \times 10^{-4}$ ]. At baseline, the BD group had a severe level of depressive symptoms [mean HDRS-21 + Atypical questions = 34.4 (14.5)] and a moderate level of manic symptoms [mean CARS-M = 15.3 (12.5)]. Severity of manic and depressive symptoms was significantly reduced at follow-up compared with entry [mean HDRS-21 + Atypical = 9.00 (8.98),  $p < 0.01$ ; mean CARS-M 3.46 (5.36),  $p = 0.01$ ]. However, more than 50% of the subjects with BD were lost to

follow-up. Many subjects had an unstable living situation upon discharge from the hospital and this may have contributed to poor follow-up. Self-report of n-3 EPA, DHA, or ALA consumption using the FFQ did not differ between the HC and BD groups at baseline [*EPA* HC: 0.03 (0.03), BD: 0.03 (0.04),  $p = 0.88$ ; *DHA* HC: 0.06 (0.07), BD: 0.04 (0.06),  $p = 0.36$ ; *ALA* HC: 0.33 (0.50), BD: 0.32 (0.65),  $p = 0.96$ ]; or in the BD group from baseline (BL) to follow-up (FU): [*EPA* BD BL: 0.02 (0.03), BD FU: 0.02 (0.03),  $p = 0.28$ ; *DHA* BD BL: 0.04 (0.05), BD FU: 0.04 (0.05),  $p = 0.22$ ; *ALA* HC: 0.28 (0.64), BD: 0.28 (0.23),  $p = 0.83$ ].

The subjects with BD predominantly were diagnosed with bipolar disorder type I ( $n = 21$ , 78%) and a smaller number were diagnosed with bipolar disorder type II ( $n = 6$ , 22%). About one-half of the subjects with BD had psychosis with the current episode, the majority ( $n = 22$ , 82%) had suicide risk, and 19% ( $n = 5$ ) had current panic disorder. At the time of assessment, more than one-half of the BD sample smoked ( $n = 14$ , 56%), 15% ( $n = 4$ ) drank above the sex-specific recommended limit within the month prior to assessment, and 39% ( $n = 10$ ) used an illicit drug within the month prior to assessment. At baseline presentation, 78% ( $n = 21$ ) of the BD subjects were taking mood-stabilizing medication, 48% ( $n = 13$ ) were taking antidepressants, and 56% ( $n = 15$ ) were taking antipsychotic medication. Treatment was not controlled for this study, and on follow-up, 69% of the subjects were taking a mood-stabilizing medication, 54% were taking an antipsychotic, and 31% were taking an antidepressant.

We report our results below categorized by fatty acid or fatty acid group. Unesterified (UE) forms of the FA were measured because they are better able to cross the blood-brain barrier (22, 23), and a difference in the UE:E ratio may signal a difference in availability of the PUFA to the brain, or a slowed production of the UE from the E plasma PUFA due to hydrolysis by circulating or tissue lipases.

Plasma n-3 concentrations, UE:E ratios, n-3 ratios, and clinical correlations

*EPA*. The mean concentration of UE EPA was lower in the BD than HC group. Although the effect size was large (Cohen's  $d = 0.86$ ), the difference bordered on statistical significance after consideration of multiple testing ( $p = 0.002$ , alpha set at 0.001). No statistically significant difference was found in the E EPA levels at baseline, nor in either form of EPA in the BD group between baseline and follow-up (Table 1). Neither mania nor depression severity correlated with UE or E EPA plasma concentrations at baseline in the BD group.



Exploratory analysis revealed that at baseline, the mean ratio of UE:E EPA was significantly lower in the BD than HC group (Table 2 and Fig. 1) ( $p = 9 \times 10^{-5}$ ), but did not differ between BD at baseline or follow-up.

Significant exploratory clinical correlations for U or E EPA or UE:E EPA ratios included a positive correlation between age and a higher mean UE EPA concentration ( $r = 0.46$ ,  $p = 0.02$ ). Panic attacks and suicidality correlated negatively and significantly with mean UE EPA plasma concentrations (panic attacks:  $r = -0.41$ ,  $p = 0.03$ ; suicidality:  $r = -0.49$ ,  $p = 0.009$ ). However, mania was positively correlated with the UE:E EPA ratio (mania:  $r = 0.51$ ,  $p = 0.006$ ).

*DHA.* No significant difference was found between UE or E DHA plasma concentrations in the BD and HC groups at baseline or between BD group at follow-up and baseline, using an adjusted alpha for multiple testing ( $p = 0.001$ ) (Table 2). Neither mania nor depression severity correlated with UE or E DHA plasma concentrations at baseline in the BD group.

Exploratory analysis revealed no differences in mean ratios of UE:E DHA at baseline, nor between BD at baseline or follow-up (Table 2).

Significant exploratory clinical correlations for U or E DHA or UE:E DHA included a positive correlation between panic attacks and UE DHA ( $r = 0.46$ ,  $p = 0.02$ ), and between panic attacks and UE:E DHA ( $r = 0.42$ ,  $p = 0.03$ ).

*ALA.* No significant difference was found between UE or E ALA plasma concentrations in the BD and HC groups at baseline or between BD group at follow-up and baseline, using an adjusted alpha for multiple testing ( $p = 0.001$ ) (Table 2). Neither mania nor depression severity correlated with UE or E ALA plasma concentrations at baseline in the BD group.

Exploratory analysis revealed no differences in mean ratios of UE:E ALA at baseline, nor between BD at baseline or follow-up (Table 2).

Significant exploratory clinical correlations for U or E ALA or UE:E ALA included a negative correlation between psychosis and E ALA ( $r = -0.46$ ,  $p = 0.03$ ).

### N-3 ratios (DHA:ALA, EPA:ALA, EPA:DHA)

Differences in the ratios of n-3 species between BD and HC, and BD at baseline and follow-up were tested in an exploratory fashion (Table 2). No group difference was found at baseline in the three ratios. At the follow-up time point, investigation of PUFA ratios revealed the UE EPA:ALA was decreased significantly ( $p = 0.002$ ), and the UE EPA:DHA increased significantly ( $p = 0.03$ ). Additionally, the E and UE DHA:ALA both decreased significantly ( $p \leq 0.001$ ).

Clinical correlations with these ratios revealed that panic attacks correlated negatively and significantly with the EPA:DHA ratio ( $r = -0.40$ ,  $p = 0.04$ ), and panic attacks were positively correlated with UE DHA:ALA ( $r = 0.47$ ,  $p = 0.01$ ).

### Plasma n-6 PUFA concentrations and clinical correlations

No significant difference was found between UE or E LA or UE or E AA concentrations in the BD and HC groups at baseline or between BD group at follow-up and baseline using an adjusted alpha for multiple testing ( $p = 0.001$ ) (Table 3). There were no significant correlations between UE or E LA, or UE or E AA and mania or depression severity.

In exploratory analysis, group differences in the UE:E LA significantly decreased between follow-up and baseline in the BD group that completed follow-up (Table 3).

Investigation for clinical correlations revealed that panic attacks were significantly positively correlated with UE AA ( $r = 0.43$ ,  $p = 0.03$ ) and mean UE:E ratios of LA ( $r = 0.42$ ,  $p = 0.03$ ) and of AA ( $r = 0.44$ ,  $p = 0.02$ ). Psychosis was negatively correlated with E LA ( $r = -0.45$ ,  $p = 0.02$ ).

### N-6 to n-3 ratio and clinical correlations

The mean ratio of E AA:EPA was significantly lower in BD compared to HC (Table 3) ( $p = 0.005$ ). There was a medium effect size difference of 0.46 calculated by Cohen's  $d$  in the group difference between HC and BD of UE:E AA:EPA, but the difference was not statistically significant ( $p = 0.10$ ). Panic ( $r = 0.59$ ,  $p =$

0.001) and psychosis ( $r = 0.43$ ,  $p = 0.03$ ) were significantly and positively correlated with UE AA:EPA ratio.

#### Saturated and monounsaturated fatty acids

No significant difference was found in saturated or monounsaturated fatty acid concentrations between BD and HC at baseline, or in BD between follow-up and baseline (see *Supplementary Table S1*). Panic attacks were positively correlated with mean concentrations of several UE fatty acids, including palmitic ( $r = 0.46$ ,  $p = 0.03$ ), palmitoleic ( $r = 0.46$ ,  $p = 0.02$ ), and oleic ( $r = 0.44$ ,  $p = 0.022$ ) acids. Psychosis was negatively correlated with mean E concentrations of palmitic ( $r = -0.53$ ,  $p = 0.004$ ) and stearic ( $r = -0.44$ ,  $p = 0.02$ ) acids.

Clinical correlations with PUFA concentrations and ratios were explored with age, sex, smoking, alcohol, current panic disorder, current suicidality, and use of antidepressant, antipsychotic, or mood-stabilizing medications. No fatty acid level or ratio correlated significantly with sex ( $p > 0.07$ ), depression ( $p > 0.14$ ), smoking ( $p > 0.06$ ), or alcohol use ( $p > 0.07$ ). There was no significant correlation between any fatty acid level and use of a medication class (antidepressant  $p > 0.24$ , mood stabilizer  $p > 0.12$ , or antipsychotic  $p > 0.08$ ).

#### Discussion

We determined differences in plasma concentrations and ratios of nine esterified (E) and unesterified (UE) long-chain fatty acids, five of which were PUFAs, between 27 BD patients and 31 HC. Two PUFAs belonged to the n-6 series, AA and its nutritionally essential shorter-chain precursor LA, three to the n-3 series, EPA, DHA, and their nutritionally essential shorter chain precursor ALA (illustrated in Fig. 2).

Based on the literature, we hypothesized that (i) plasma concentrations of n-3 PUFA would be lower and concentrations of n-6 PUFA would be higher in patients with BD with manic or depressive symptoms; (ii) n-3 PUFA would increase and n-6 PUFA decrease after naturalistic treatment of the patients; and (iii) lower n-3 and higher n-6 PUFA concentrations would be associated with manic and depressive symptoms, regardless of medication status.

After correcting for 40 comparisons, we did not find a significant difference between the plasma E or UE concentration of any of the fatty acids, although the effect size indicating a reduced EPA in BD compared with HC was high and the p-value was 0.002, suggesting a robust effect. This suggestion is particularly relevant because this study should be considered exploratory in a unique patient cohort, and the multiple comparison restriction should be taken with reservations considering the likelihood of a Type 2 error (35).

Some evidence indicates that EPA is the active n-3 PUFA responsible for mood-altering effects in n-3 PUFA supplementation studies of depression (36, 37). Low EPA may be a vulnerability factor to experiencing mood episodes, and alteration of its elongation from ALA may influence effective treatment. Other studies of patients with BD also have reported reduced plasma EPA (38), and efficacy of nutritional supplementation with EPA (27, 39), although the issue is far from settled.

EPA metabolites include bioactive cyclopentenone-IsoP molecules, termed A(3)/J(3)-IsoPs, formed by EPA peroxidation (40), and anti-inflammatory resolvin E1 (41). EPA has positive effects in mitochondria (42), a protective effect in cardiac myocytes (43), and a regulatory effect on brain capillary tight junctions (44) and other capillary endothelium (45). In this regard, while unesterified EPA is largely  $\beta$ -oxidized after entering rat brain, as compared with the incorporation of intact DHA into brain membrane phospholipids (46), EPA's mechanism of action in BD may be at the capillary endothelium, which has a high mitochondrial content (47) and may be disturbed due to neuroinflammation in BD (48).

Lower n-3 PUFA have been seen in a number of clinical studies of BD, particularly studies that investigate erythrocyte concentrations. McNamara et al. (19) found differences in erythrocyte EPA + DHA levels, and three studies reported lower erythrocyte DHA in symptomatic BD groups (18, 19, 49). However, Sublette et al. (17) reported on plasma concentrations of UE and E PUFA, and found no between-group differences in UE PUFA levels or the UE AA:EPA ratio. The critical differences may lie in the different methods of measuring PUFA, in the different symptomatic states of the subject populations, and in the different dietary intake status.

While plasma PUFA concentrations may vary more over time than erythrocyte concentrations, due to the four-month mean life time of circulating erythrocytes, studying plasma concentrations allows us to

evaluate circulating UE and E PUFA separately. Additionally, plasma concentrations reflect immediate consequences of changing diet and liver metabolism. Esterase activity on circulating lipoproteins releases UE PUFA, the form that preferably enters the brain, likely by a diffusional mechanism (50, 51). After entering brain, the largest percentage of UE shorter-chain PUFA (e.g. LA, ALA, EPA) is rapidly beta-oxidized by mitochondria (38), whereas the elongated AA and DHA forms preferentially enter the *sn*-2 position of brain membrane phospholipids to replace the respective PUFAs that have been hydrolyzed and lost by metabolism.

The suggested beneficial effects of certain PUFAs may be peripheral as well as central. Increased UE fatty acids triggered by fasting have been shown in rodents to drive immunosuppression (52). Alternately, conversion of EPA to DHA in the liver (46) may drive the treatment effect. One intervention study using UE PUFA showed that administration of fish oil increased brain UE DHA and a neuroprotective DHA metabolite, neuroprotectin D-1, in a rodent model of Alzheimer's disease (54). Additionally, phospholipase A<sub>2</sub> genes and enzyme levels may differ between BD and HC groups, which may affect production of plasma PUFA (52-58). A recent trial of n-3 PUFA for reduction of plasma triglycerides showed a phospholipase A<sub>2</sub> genotype x treatment interaction (59). Therefore investigation of UE and E PUFA provides a different window of investigation than of PUFA within RBC membrane, into the role of PUFA metabolism in BD.

#### Changes at follow-up in n-3 PUFA metabolism

We engaged participants during an active mood episode, and monitored symptoms during recovery, asking them to return when they no longer met threshold criteria for mania and depression. Treatment was not altered from usual care during this time. During this follow-up period, we lost approximately half of our participants due to inability to contact them. Therefore, our results in investigative comparisons between baseline and follow-up are exploratory and only suggestive due to the small sample size. We found no difference in plasma levels of PUFA when comparing mean baseline concentrations to concentrations at follow-up, however ratios of some plasma n-3 PUFA concentrations were significantly different at follow-up from baseline in BD subjects. There was no change in self-reported n-3 PUFA intake at follow-up. The ratio changes indicate *decreases* in UE DHA relative to UE ALA, in UE EPA relative to UE ALA, and in E DHA relative to E ALA. Since ALA once ingested can be elongated and desaturated, particularly in the liver, first to EPA and then to DHA (60), these results

suggest some limitation to these metabolic conversions. One possibility is drug effects on the liver (61). Though we cannot comment on mechanism from our data, the ratio data suggest abnormal metabolic relations among the three measured n-3 PUFAs.

#### Symptom and clinical correlations with fatty acid levels and ratios

Symptom severity has been correlated with circulating PUFA biomarkers in the Sublette et al.(17) and Evans et al. (20) studies, but not in the Chiu et al. (18) and McNamara et al. (19) studies. Sublette et al. found a positive correlation between manic symptoms and the UE AA:EPA ratio. In our study, however, the UE AA:EPA ratio was not correlated with mania, but was positively correlated with psychosis. Two factors that could affect this ratio differed in our trial from the Sublette trial, including the symptomatic state of our subjects (we had few subjects with pure mania in our study), and including patients currently on psychiatric medication. We found the UE:E ratio of EPA was positively correlated with mania, which would not be expected given that a lower UE:E EPA was found in BD compared to HC. This will bear further investigation.

In our study, subjects with panic attacks as part of the current mood episode showed lower n-3 and higher n-6 concentrations, and a higher n-6 to n-3 ratio. This pattern suggests activation of an inflammatory pathway or reduction of an anti-inflammatory pathway. The noradrenergic surge seen in panic attacks has been hypothesized to be related to the dopaminergic surge seen in mania in BD, and perhaps panic identifies a subset of mania or is a severity marker of psychiatric illness (62-65). Together, ours and others' findings suggest an altered PUFA metabolism in BD, in accord with pre-clinical data.

We found a positive correlation between UE saturated fatty acids and panic, and a negative association between E saturated fatty acids and psychosis. The differentiating factor may be the esterification status, as the UE form preferentially enters brain. UE palmitic acid was shown to increase anxiety-like behaviors and immunosuppression in rodent models (66, 67). UE fatty acids may be associated with clinical phenotype through an immunological link.

Medication status

While both Sublette et al. (17) and McNamara et al. (19) studied PUFA in un-medicated patients, the Chiu et al. study (18) found no difference between un-medicated and medicated patients. Our approach differed, as we studied relations between effective treatment and biological markers. We included patients who were ill though medicated, and those with comorbid psychiatric illness, specifically looking for differences before and after treatment. We had no un-medicated subjects in our sample, but we did not find that concentrations or ratios of PUFA were correlated with antidepressant, antipsychotic, or mood stabilizers as medication groups.

#### Dietary report

We found no correlations between self-report of ALA, EPA, and DHA intake and plasma fatty acid levels, which could occur for several reasons. The subjects in this study may have been unable to accurately fill out the questionnaire given the severity of mood symptoms. A more controlled dietary regimen would be useful in this regard (68). Additionally, individual metabolic differences may mask correlations with reported intake. The memory difficulty inherent in mood episodes may have interfered with the ability to report food intake accurately. Further study with this instrument would be beneficial to understand barriers to its use.

#### Limitations

Limitations include the use of patients who are taking medication in a study of biomarkers. While the heterogeneity of the subjects with BD with regard to medication use at baseline, in follow-up and comorbid psychiatric illness may limit the precision with which the findings of PUFA differences can be related to bipolar neuropathology, this study design maximizes the generalizability of the findings to a real-world clinical population.

If EPA metabolism differs in patients who are symptomatic and is altered with successful treatment, regardless of the medication class, this suggests that effective medications in practice have a similar biological impact on the n-3/n-6 metabolic balance, mirroring pre-clinical findings (12). Additionally, our results at follow-up are only suggestive, as (i) treatment was not delivered as a controlled intervention, and (ii) less than 50% of the sample completed the follow-up. While baseline factors did not differ between the groups that did or did not follow-up, we could not measure treatment response in those

who did not complete the study. The patients had a high rate of unstable housing and contact information, which may have been a factor in their inability to complete the study. Other limitations include self-report of dietary n-3 input only. Baseline dietary intake of PUFA may indeed influence response of the system to intervention. In addition, this study was not large enough to look for subgroups of subjects who may have PUFA alterations, while others may not.

## Conclusions

We found (i) a lower plasma UE:E concentration ratio of EPA, (ii) a trend toward lower U EPA in the symptomatic BD state, and (iii) altered n-3 PUFA ratios upon follow-up in a subset of the BD sample. Altered plasma circulating n-3 PUFA concentrations may influence vulnerability to a mood state, and altered n-3 PUFA ratios could indicate changes in PUFA metabolism concurrent with symptom improvement. Panic attacks, which may be a marker of clinical severity, were correlated with lower n-3 and higher n-6 PUFA concentrations and a higher n-6 to n-3 ratio. Our findings are consistent with preclinical and postmortem data and suggest testing interventions that increase n-3 and decrease n-6 dietary PUFA intake.

## Implications

While initial n-3 PUFA supplementation studies in BD demonstrated efficacy in open trials (69-72), and subsequent randomized controlled trials (73, 74) failed to replicate these findings (75-79), preclinical and post-mortem data implicate PUFA in pathophysiology of BD (12, 13). Interpretation of the varied responses seen in randomized, controlled trials is confounded by a number of factors, including heterogeneity of diagnosis, design of trials, compliance to study drug, and composition and dose of supplements (36, 80-84).

One reason that supplement studies may have not shown effect may be that in the past century, the n-6 PUFA precursor LA has increased in the average US diet, resulting in decreasing tissue concentrations of n-3 EPA and DHA and increasing concentrations of AA and LA and their metabolites (85–87). Addition of an n-3 supplement without concurrent reduction in dietary n-6 LA may not produce clinically meaningful benefit. A recent randomized trial investigated PUFA dietary intervention in chronic migraine headache, a condition with considerable overlap with BD in pathophysiology, comorbidity, and treatment (34, 88-



101). Clinical efficacy and biochemical effects of a high n-3 EPA + DHA plus low n-6 LA (H3-L6) diet were compared to a low n-6 PUFA diet (L6). The H3-L6 group experienced a significantly greater reduction in headache hours per day, headache days per month, headache-related quality-of-life and psychological distress than the L6 group, though both groups experienced improvement from the run-in phase (68). Interestingly, both the L6 and the H3-L6 groups had increases in UE EPA, specifically, among PUFA (102). Based on the clinical and neuroinflammatory links between BD and migraine, concurrent lowering of dietary n-6 in BD may also be necessary for effective treatment of bipolar disorder with n-3 PUFA supplementation (103). Future studies of the specific signaling pathways and lipid mediators linking n-3 and n-6 PUFA to BD pathogenesis may lead to development of targeted dietary and medication strategies for treating BD.

### Figure legends

*Fig. 1.* At baseline, the mean ratio of unesterified (UE) to esterified (E) concentration of eicosapentaenoic acid (EPA) was significantly lower in the bipolar disorder group when compared to the healthy control group. CI = confidence interval.

*Fig. 2.* The metabolic pathway of the polyunsaturated fatty acids linoleic acid (the n-6 pathway) and alpha-linolenic acid (the n-3 pathway) to arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

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Table 1. Demographics

	Healthy controls (n = 31)	Bipolar disorder (n = 27) <sup>a</sup>	p-value
Sex, female, n (%)	18 (60)	12 (44)	0.30
Race, n (%)			
Asian	4 (13)	0 (0)	0.19
Black	1 (3)	1 (3.7)	
White	26 (84)	25 (93)	
Mixed	0 (0)	1 (3.7)	
Ethnicity, n (%)			0.18
Hispanic	2 (6)	0 (0)	
Not Hispanic	29 (94)	27 (100)	
Marital status, n (%)			0.03
Married	16 (52)	5 (19)	
Separated	1 (3)	1 (3)	
Divorced	0 (0)	3 (11)	
Never married	14 (46)	18 (67)	
Employment, n (%)			< 0.01
Unemployed	2 (6)	14 (54)	
Disabled	0 (0)	5 (19)	
Employed	17 (55)	6 (23)	
Student	11 (36)	1 (4)	

Retired	1 (3)	0 (0)	
Smoking, n (%)	1 (3)	14 (56)	NA
Heavy alcohol use, n (%)	1 (3)	4 (15)	
Drug use, n (%)	1 (3)	10 (39)	
Suicidality, n (%)	0 (0)	22 (82)	
Psychosis, n (%)	0 (0)	14 (52)	
Panic, n (%)	0 (0)	5 (19)	
Age, years, mean (SD)	32 (11)	35 (11)	0.31
BMI, mean (SD)	24.9 (4.7)	30.5 (6.0)	$1.7 \times 10^{-4}$
Mania (CARS-M), mean (SD)	15.3 (12.5)	0 (0)	
Depression (HDRS-21 + Atypical), mean (SD)	34.4 (14.5)	0.3 (0.6)	

SD = standard deviation; BMI = body mass index; CARS-M = Clinician-Administered Rating Scale for Mania; HDRS = Hamilton Depression Rating Scale-21.

<sup>a</sup>At baseline.

Table 2. N-3 polyunsaturated fatty acid plasma concentrations and ratios in the bipolar disorder and healthy control groups at baseline and follow-up (nmol/mL)

	Baseline			Bipolar disorder, follow-up		
	Healthy controls (n = 31)	Bipolar disorder (n = 27)	p-value	Baseline (n = 13)	Follow-up (n = 13)	p-value
	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	
<b>ALA</b>						
E	19.89 (25.64)	18.86 (12.81)	0.85	18.48 (17.30)	25.45 (15.42)	0.18
UE	2.10 (2.80)	1.40 (1.77)	0.27	0.88 (1.06)	2.28 (1.72)	0.02
<b>DHA</b>						
E	220.93 (93.79)	221.37 (83.10)	0.99	227.46 (84.10)	177.71 (161.19)	0.28
UE	1.07 (0.64)	1.04 (1.04)	0.89	0.73 (0.38)	0.55 (0.26)	0.11
<b>EPA</b>						
E	8.91 (6.94)	13.91 (13.39)	0.07	14.62 (18.98)	12.68 (6.86)	0.75
UE	0.32 (0.18)	0.20 (0.08)	0.002	0.21 (0.06)	0.31 (0.26)	0.16
<b>UE:E</b>						

ALA	0.14 (0.13)	0.08 (0.10)	0.05	0.08 (0.13)	0.12 (0.10)	0.27
DHA	0.006 (0.005)	0.005 (0.003)	0.34	0.004 (0.003)	0.005 (0.003)	0.32
EPA	0.05 (0.03)	0.02 (0.01)	$9 \times 10^{-5}$	0.02 (0.02)	0.04 (0.07)	0.24
<b>DHA:ALA</b>						
E	18.20 (11.34)	14.27 (6.05)	0.10	16.15 (6.05)	7.44 (5.36)	0.001
UE	0.92 (0.86)	1.13 (0.77)	0.33	1.12 (0.43)	0.31 (0.17)	$4 \times 10^{-5}$
<b>EPA:ALA</b>						
E	0.61 (0.23)	0.71 (0.18)	0.07	0.72 (0.19)	0.54 (0.20)	0.06
UE	0.28 (0.18)	0.26 (0.17)	0.78	0.36 (0.17)	0.19 (0.16)	0.002
<b>EPA:DHA</b>						
E	0.05 (0.03)	0.08 (0.12)	0.13	0.09 (0.17)	0.10 (0.07)	0.86
UE	0.39 (0.29)	0.30 (0.21)	0.18	0.37 (0.24)	0.58 (0.29)	0.03

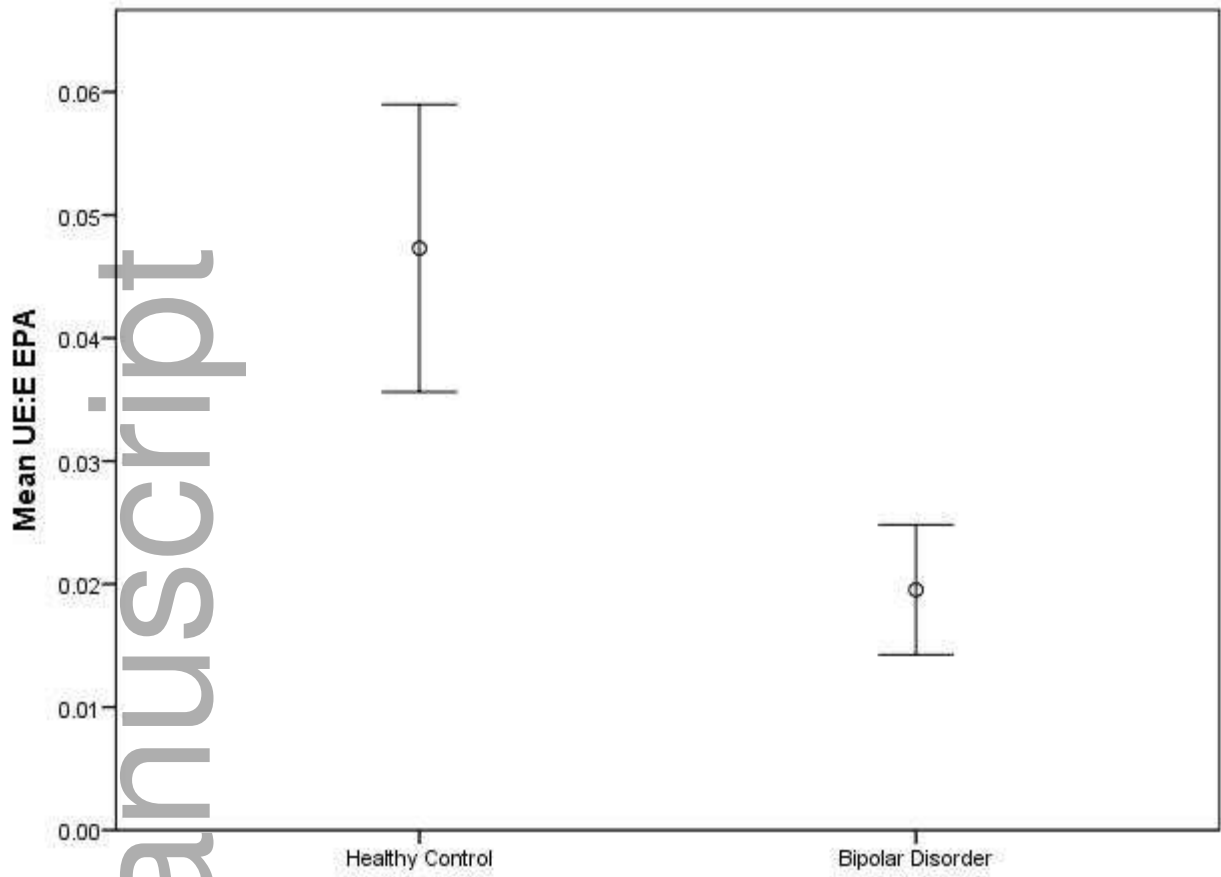
SD = standard deviation; E = esterified; UE = unesterified; ALA = alpha-linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid.

Table 3. N-6 and n-6:n-3 polyunsaturated fatty acid plasma concentrations and ratios in the bipolar disorder and healthy control groups at baseline and follow-up (nmol/mL)



	Baseline			Bipolar disorder, follow-up		
	Healthy controls (n = 31)	Bipolar disorder (n = 27)	p-value	Baseline (n = 13)	Follow-up (n = 13)	p-value
	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	
<b>LA</b>						
E	2684.88 (794.62)	2624.80 (638.07)	0.75	2548.78 (724.90)	3808.82 (2030.68)	0.02
UE	54.07 (30.18)	62.81 (57.70)	0.48	53.45 (47.66)	36.66 (27.46)	0.07
<b>AA</b>						
E	676.94 (202.42)	720.07 (196.35)	0.42	739.90 (244.02)	950.11 (449.19)	0.03
UE	2.73 (1.64)	2.85 (2.15)	0.82	2.28 (1.67)	1.98 (0.84)	0.46
<b>UE:E</b>						
LA	0.02 (0.01)	0.03 (0.02)	0.39	0.02 (0.02)	0.01 (0.01)	0.02
AA	0.004 (0.002)	0.004 (0.003)	0.85	0.003 (0.002)	0.002 (0.001)	0.18
<b>AA: EPA</b>						
E	100.69 (55.32)	68.05 (26.48)	0.005	78.68 (31.02)	88.05 (39.86)	0.54
UE	10.96 (7.59)	18.59 (22.13)	0.10	11.83 (9.62)	9.65 (7.18)	0.28

SD = standard deviation; E = esterified; UE = unesterified; LA = linoleic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid.



Error Bars: 95% CI

bdi\_12337\_f1.tiff

