Prospective clinical trial examining the impact of genetic variation in *FADS1* on the metabolism of linoleic acid- and gamma-linolenic acid-containing botanical oils

Susan Sergeant[1,2], Brian Hallmark[3], Rasika A. Mathias[2,4], Tammy L. Mustin[2,5], Priscilla Ivester[2,5], Maggie L.Bohannon[2,5], Ingo Ruczinski[2,6], Laurel Johnstone[3], Michael C. Seeds[2,7] and Floyd H. Chilton[2,3,*]

[1]Department of Biochemistry, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157 USA. E-mail: ssergean@wakehealth.edu(SS)

[2]Center for Botanical Lipids and Inflammatory Disease Prevention, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157 USA. (SS, RAM, TLM, PI, MLB, IR, MCS, FHC)

[3]BIO5 Institute, 1657 E Helen St., University of Arizona, Tucson, AZ, 85719 USA. Email: bhallmark@statlab.bio5.org; laureljo@email.arizona.edu (BH, LJ, FHC)

[4]Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University, Baltimore, MD 21224 USA. Email: rmathias@jhmi.edu (RAM)

[5]Department of Physiology/Pharmacology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157 USA. E-mail: talee@wakehealth.edu;. ivester@wakehealth.edu; mlbohann@wakehealth.edu (TLM, PI, MLB)

[6]Bloomberg School of Public Health, Department of Biostatistics. 615 N. Wolfe Street, Baltimore, MD 21205 USA. Email: iruczin1@jhu.edu (IR)

[7]Department of Internal Medicine, Section on Molecular Medicine, Wake Forest School of Medicine, Medical Center Blvd. Winston-Salem, NC 27157 USA.
E-mail: mseeds@wakehealth.edu (MCS)

*Corresponding Author: Floyd H. Chilton, PhD. Department of Nutritional Sciences and the BIO5 Institute, BSRL Building, 1230 N. Cherry Ave. Tucson, AZ, 85719 USA.520-621-5327.fchilton@email.arizona.edu

Running Head: *FADS* variants impact GLA metabolism

Conflict of interest statements: No authors have a conflict of interest.

Sources of Support: This work was supported by grants from the National Institutes of Health, P50 AT002782 (FHC), R01 AT008621 (FHC) and UL1TR001420 (Wake Forest CTSI).

Clinical Trial Registry: Clinicaltrial.gov NCT02337231

Abbreviations: ARA, arachidonic acid; BMI, body mass index; BO, borage oil; CRP, C-reactive protein; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid**;** DPA, docosapentaenoic acid**;** EPA, eicosapentaenoic acid**;** FAME, fatty acid methyl esters; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; GLA, gamma-linolenic acid; HODE, hydroxyoctadecadienoic acid; LA,linoleic acid; LC, long

chain; n-3, omega-3; n-6 omega-6; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SO, soybean oil; TX, thromboxane

De-identified data described in the manuscript and analytic code will be made available upon request pending application and approval.

ABSTRACT

Background

Unexplained heterogeneity in clinical trials has resulted in questions regarding the effectiveness of gamma-linolenic (GLA)-containing botanical oil supplements. This heterogeneity may be explained by genetic variation within the fatty acid desaturase (*FADS*) gene cluster that is associated with circulating and tissue levels of arachidonic acid (ARA) and dihommo-gamma linolenic acid (DGLA), both of which may be synthesized from GLA and result in pro-inflammatory and anti-inflammatory metabolites, respectively.

Objectives

The objective of this study was to prospectively compare the capacity of a non-Hispanic White cohort, stratified by *FADS* genotype at the key SNP rs174537, to metabolize 18-carbon n-6 PUFAs in borage oil (BO) and soybean oil (SO) to GLA, DGLA and ARA.

Design

Healthy adults (N=64) participated in a randomized, double-blind, cross-over intervention. Individuals received encapsulated BO (*Borago officinalis;* 37% LA, 23% GLA) or SO (*Glycine max* (L.) Merr.; 50% LA, 0% GLA) for 4 weeks, followed by an 8-week washout period, before consuming the opposite oil for 4 weeks. Serum lipids and markers of inflammation (C-reactive protein) were assessed at baseline and during the second and fourth weeks of intervention for both oil types.

Results

SO supplementation failed to alter circulating levels of any n-6 LC-PUFAs. In contrast, a modest daily dose of BO elevated serum levels of GLA and DGLA in a rs174537 genotype-dependent manner. In particular, DGLA increased by 57% (95% CI: (0.38, 0.79)) in GG individuals, but by 141% (95% CI: (1.03, 2.85)) in TT individuals. For ARA, baseline levels varied substantially by genotype and increased modestly with BO supplementation suggesting a key role for *FADS* variation in the balance of DGLA and ARA.

Conclusions

The results of this study clearly suggest that personalized- and population-based approaches considering *FADS* genetic variation may be necessary to optimize the design of future clinical studies with GLA-containing oils.

Keywords: polyunsaturated fatty acids (PUFAs), n-3 fatty acids, n-6 fatty acids, gene-diet interaction, gamma-linolenic acid, borage oil, randomized cross-over design, soybean oil, arachidonic acid, precision nutrition

INTRODUCTION

Gamma-linolenic acid (GLA, 18:3) is an omega-6 (n-6), 18-carbon polyunsaturated fatty acid (PUFA) found in several seed oils including Evening Primrose (~10% of total fatty acids) and Borage (~21%). While many types of studies (*in vitro*, *in vivo,* animal and human) have shown that GLA-containing oils reduce inflammatory processes and impact inflammatory diseases (rheumatoid arthritis, asthma and atopic dermatitis (1-10)), there is large unexplained heterogeneity in clinical studies, and recent meta-analyses have questioned the efficacy of these oils (11-15). The putative molecular mechanisms by which GLA-containing oils act appears to originate in large part from its metabolic elongation (by *ELOVL5* encoded enzymatic activity) to dihomo-gamma linolenic acid (DGLA, 20:3n6); **Figure 1**(16-18). Oxidative metabolites of DGLA have anti-inflammatory, anti-proliferative, anti-atherogenic and vasodilation effects (18-30). DGLA can be further metabolized to arachidonic acid (ARA, 20:4n6) through a ∆-5 desaturase step encoded by the *FADS1* gene within the *FADS* cluster. In contrast to DGLA, ARA-derived bioactive lipids are generally pro-inflammatory (26, 31). These factors may contribute to the complexity of interpreting results gathered from GLA supplementation studies regarding their impact on inflammatory based diseases.

The ∆-6 (FADS2) and ∆-5 (FADS1) desaturation steps (**Figure 1**), which form GLA from LA, and ARA from DGLA, respectively, were once assumed to have uniform metabolic efficiency across individuals and populations. However, considerable evidence now indicates that common genetic and epigenetic variations in the *FADS1/2* genes markedly impact the overall rate of conversion of LA to ARA. In fact, the strongest *FADS* genetic associations observed to date are for ratios of ARA- to DGLA-

containing lipids (a surrogate measure for FADS1 activity), with minor alleles consistently associated with decreased ARA and increased DGLA levels (32-34). Importantly, the frequency of *FADS* cluster variants differ dramatically among different global populations (33, 35-44).

Together, these findings raise important questions regarding the impact of supplementation with GLA-containing oils in diverse populations. Importantly, they suggest that a generalized “one size fits all” dietary supplementation approach may not be appropriate or safe. A key hypothesis arising from previous studies is that the impact of GLA consumption and its subsequent metabolism depend on *FADS1* variant–associated metabolic efficiency. Specifically, an inefficient conversion phenotype would result in the accumulation of DGLA-containing lipids. In contrast, the efficient converter phenotype, would exhibit increased ARA-containing lipids at the expense of DGLA-containing lipids. We tested this hypothesis utilizing a prospective, cross-over efficacy trial of GLA supplementation in a European-ancestry population stratified by the *FADS* genotypes at rs174537.

SUBJECTS AND METHODS

Study Overview

This study was designed to examine how GLA supplementation changes serum fatty acid levels, in particular levels of GLA, DGLA and ARA, and to determine whether the changes vary by genotype at rs174537. We employed a double-blinded, randomized, cross-over study design using two botanical oils – borage oil (BO), a source of GLA, as the treatment, and soybean oil (SO), which lacks GLA but contains

LA, as the control (45). In this design, illustrated in **Figure 2B**, all subjects received both oils, albeit in random order. Biospecimens (fasting blood and urine), vital signs (blood pressure and resting heart rate) and morphometric measurements (waist and hip circumference, height; body mass index [BMI] and percent body fat) were obtained at baseline, two and four weeks for each supplementation phase. An eight-week washout period was used between the two oil supplementation periods. The primary outcomes were levels of circulating GLA, DGLA and ARA.

Study Subjects

This study was conducted in the Wake Forest Baptist Medical Center (WFBMC), and was reviewed and approved by the Wake Forest University Health Sciences Institutional Review Board (IRB) and NIH/OCRA. The inclusion criteria were healthy, self-identifying non-Hispanic White adults of European ancestry, aged 21 to 65 years old, free of major diseases and previously genotyped at rs174537 through a concurrent IRB-approved screening study. Participant exclusion criteria included the following: a) current use of anti-inflammatory drugs (NSAIDs, oral/IV steroids, other injection anti-inflammatory drugs, >100mg aspirin per day, leukotriene receptor antagonists), niacin, fibrates or fish oil; b) blood pressure >130/90mm/Hg; c) fasting blood triglycerides >150mg/dL, fasting blood glucose > 125mg/dl; d) having a pacemaker, defibrillator or myocardial infarction, vascular surgery, or stroke in the preceding year; e) any stage heart failure, prior cholecystectomy, end stage renal disease; f) BMI <19 or >30; g) pregnancy; h) alcohol use >14 drinks per week; i) self-reported current tobacco smoking or other illicit drug use; and j) intolerance or allergy to BO or SO. A summary of the

recruitment and enrollment numbers for the 16-week study is shown in **Figure 2A**. Written informed consent was obtained from all subjects at an initial screening visit. Participants were asked to report any change in their medical condition at each study visit.

Randomization, Compliance and Dropout

Consented participants were stratified by genotype at rs174537 and randomized within strata into one of the two oil consumption sequences (SO followed by BO [Arm A] or BO followed by SO [Arm B]) as shown in **Figure 2B**. Block randomization was used to ensure approximately equal accrual to each sequence from each stratum over time. The study staff and participants were blinded to both genotype and oil supplement assignment.

Compliance was monitored by multiple mechanisms including serum fatty acid profiles at two and four weeks of the intervention, the capsule log and counts of returned capsules at each visit. Subjects used a log to record intake of study oils on a daily basis. Returned capsule counts indicated that an average of 94.6% of capsules was consumed as directed. Only four participants (6.3%) consumed less than 80% of their oil supplements.

Dietary Oil Supplements

The oils used in this study were obtained from borage seeds (*Borago officinalis* L.; 23% GLA, 37% LA) and soybeans (*Glycine max* (L.) Merr.; 0% GLA, 50% LA) and

were generously supplied by Nordic Naturals (Watsonville, CA, USA). Subjects in each arm consumed a total of 10 oil capsules (1.2g oil/capsule) daily with half consumed at morning and evening meals. As shown in **Table 1** (right two columns), the LA dosage provided by the SO (control arm) and BO (experimental arm) capsules were 5.27g/day and 4.11g/day, respectively. The GLA dosage provided by the SO and BO capsules were 0 g/day and 2.54g/day, respectively. For comparison, supplemental GLA has been safely administered in clinical trials at oral doses of 2.8 grams per day or less, for up to a year. The physical appearance of the soft gelatin oil capsules was indistinguishable. Participants were advised to consume their normal diet but to minimize fish intake during the supplementation period, refrain from self-medication with anti-inflammatory drugs, and inform the study staff about any health concerns during the study. The same dietary advice was given for the 8-week washout period.

A significant due diligence process was undertaken to identify high quality oil products from reliable vendors (46). All relevant vendor-derived product documentation was obtained and archived. The results of in-house fatty acid analyses found profiles of the oil products to be comparable to that stated in vendor documentation. **Table 1** shows the fatty acid composition (derived from in-house analyses) of each oil and the daily dose of these fatty acids. **Figure 1** shows the primary entry points where target PUFAs (LA in soybean oil [SO] and borage oil [BO]; GLA in BO) in these oil supplements enter the LC-PUFA biosynthetic pathway.

Prior to final oil product approval and encapsulation, the oils were authenticated and subjected to thorough evaluation (fatty acid profile, oxidation status, endogenous and exogenous contaminants) by the Botanical and Quality Assurance Core Lab within

the Wake Forest School of Medicine Center for Botanical Lipids and Inflammatory Disease Prevention. The quality of the encapsulated oil products was monitored (fatty acid profile and oxidation status) over the course of the study. Archival samples have been maintained. Thus, the study products and associated documentation were obtained, analyzed, inventoried, dispensed and archived with the same rigor typical of that used for drug studies with the goal of generating reproducible results from the clinical trial (47, 48).

Fatty Acid Analysis

Total serum fatty acids were analyzed from fasting blood, and levels of GLA, DGLA and ARA were the primary outcome measures for this study. Fatty acid methyl esters (FAME) were prepared (49) after alkaline hydrolysis of complex lipids in duplicate samples (100µl) in the presence of an internal standard (triheptadecanoin: Nuchek Prep, Elysian, MN, USA; included for purposes of fatty acid quantification) as previously described (50, 51). A standard panel of 26 fatty acids (which accounted for 99% of the fatty acids in the samples) was quantified by gas chromatography with flame ionization detection (GC-FID) using an HP5890 instrument with DB-23 column (30m, 0·25 mm ID, 0·25 µm film) fitted with an inert pre-column (1 m, 0.53 mm ID) for cool on-column injection. The instrument response factor has been calculated based on the use of external standard sets for quality assurance purposes and a mixture of known FAMEs was run with each sample set to monitor instrument performance (45). Individual fatty acids are expressed as percent of total fatty acids in each sample.

The fatty acid composition of the oil supplements (**Table 1**) was determined in aliquots of oil quantitatively dilutions (in hexane) and processed as described above in the presence of the internal standard. For the purposes of product authentication, individual fatty acids in oil products are expressed as area % (**Table 1**) to facilitate comparison to vendor documentation of product fatty acid profile. The daily fatty acid doses derived from each oil were calculated from units of grams fatty acid/gram oil. The integrity of the encapsulated oils was monitored over time by routine evaluation of fatty acid content and oxidation status to ensure that a high-quality product was provided to participants. For the oxidation status of oils, standard food industry assays were employed. These included peroxide value (PV; primary oxidants; iodometric titration assay (52); European Pharmacopeia 2.5.5), anisidine value (AV; secondary oxidants; colorimetric assay (53); European Pharmacopeia 2.5.36), and Totox (total oxidation = 2PV+AV).

Biochemical Measurements

Routine cardiometabolic and inflammatory biomarkers were measured as secondary outcomes. Serum derived from fasting blood was used to measure glucose (enzymatic assay), high-sensitivity C-reactive protein (hsCRP; immunochemoluminometric assay), and serum lipids (total cholesterol, triglycerides, HDL-, VLDL- and LDL-cholesterol; enzymatic assays). Urinary creatinine levels were measured (spectrophotometric assay) in order to normalize data of future urine analyses. An assessment of hepatic function (7 analyte panel measured in serum) was performed only at the screening stage and used to evaluate healthy baseline screening

status. The hepatic function tests conducted included alkaline phosphatase (kinetic assay), albumin (colorimetric assay), total protein (colorimetric assay), total and direct bilirubin (colorimetric assay), and aspartate (SGOT) and alanine (SGDT) aminotransferase (kinetic assays). These endpoints were analyzed by a qualified clinical laboratory (Lab Corp, Burlington, NC)

Data Analysis

All statistical analyses were conducted in R version 3.5.0 (54). For each subject, we examined change in measured fatty acids (FAs) as % of total FAs over time, focusing on LA, GLA, DGLA and ARA as illustrated in **Figure 1** and their ratios (GLA/LA, DGLA/GLA, and ARA/DGLA), which serve as surrogates for the (steady-state) enzymatic activity along the steps of the LC-PUFA biosynthetic pathway. We additionally examined the important n-3 LC-PUFAs: EPA, DPA and DHA.

The distribution of each FA was first examined for outliers, and the values plotted over time for each subject. Values for individual FAs are relative abundances (% of total FA) and hence are compositional in nature. For the statistical models, FA outcomes were first log-ratio transformed (i.e., $x_{new} = \log(x/1-x)$, where x is a FA proportion) as is appropriate for compositional data (55). All model results were back-transformed to percent of total FAs for interpretation and presentation.

Baseline FA values were stratified by genotype and compared using a linear model with the FA as outcome, genotype as the explanatory variable with age and sex also included. The washout period was evaluated by comparing FA measurements from

each individual at the beginning of each oil supplementation period (i.e., V2 and V5) with a paired t-test. To assess how responses to supplementation over time varied by genotype, for each FA, we fit a linear mixed-effects model with a random intercept for each subject. Several choices for the correlation structure of the repeated measurements were compared using likelihood ratio tests, and a heterogeneous autoregressive AR (1) model was used based on those results. The main explanatory variables in the models were rs174537 genotype under an additive model (i.e., 0,1, or 2 copies of the T allele), data collection time period (0, 2, or 4 weeks), oil type (BO vs SO), as well as their interactions. As FA levels can vary by age and sex, those variables were included as covariates. A term for study arm (oil sequence) was also included, but was consistently found to have small and non-significant estimated effects. The mixed-effects models were fit using the nlme R package (56) with maximum likelihood (ML) used for model comparison and restricted ML (REML) used for parameter estimation.

For another perspective on the data, in addition to the mixed-model analyses, we also looked at fold-change from baseline in subjects that completed both arms of the study (n=53; **Table 4**). For each subject, fold changes from baseline values were computed for the four FAs illustrated in **Figure 1** (i.e., LA, GLA, DGLA and ARA) as well as for the n-3 LC-PUFA EPA, DPA, and DHA. A linear model was then used to assess the effects of supplementation with oil examined separately. For each FA, a linear regression was performed with log-fold change from baseline as the outcome and genotype as the primary covariate (additive genetic model), adjusted for age, sex, and study arm.

Due to the large number of hypothesis tests conducted herein (10 FAs or FA ratios x ~10 tests = ~100 tests), we adopted a conservative threshold of p=0.05/100 = 0.0005 for statistical significance. Furthermore, following recent recommendations of the statistical community (57), we avoided simple dichotomization of results based on a significance threshold, and instead focused on estimates and confidence intervals, and their biological significances. The CONSORT RCT reporting guidelines were used as a checklist reporting tool for this study (58).

RESULTS

Characteristics of the Participant Population

As shown in **Figure 2A**, n=73 (80%) of the 91 consented individuals were eligible to participate in this study. Of the consented participants, n=66 of those individuals were randomized and enrolled in the intervention. Based on an Intent-to-Treat analysis, data from 64 participants was used in the final analyses. At the recommendation of the WFBMC Research Integrity Office, two participants were removed from all analyses due to conflicting information regarding their randomization and treatment. Information indicated that in those two cases, the treatments were partially or completely reversed. As the primary goal of this study was to estimate changes in FA levels due to BO supplementation by genotype, deliberate inclusion of incorrect treatment data would bias the estimates of interest. For this reason, we employed a modified intention-to-treat analysis with a final sample size of n=64 subjects. The characteristics of this study population are detailed in **Table 2**. Of the participants that began the intervention, a total

of n=11 participants did not complete the intervention. Reasons for dropping out included scheduling conflicts (n=3); elevated C-reactive protein on two occasions (n=3); the need to use non-steroidal anti-inflammatory drug (NSAID) medications (n=2); gastrointestinal upset (n=1); difficulty swallowing oil capsules (n=1); and pregnancy (n=1).

Baseline Differences in PUFAs and LC-PUFAs by rs174537 Genotype

In agreement with previous studies, there were significant baseline (non-supplemented) differences by rs174537 genotype in the levels of ARA and the fatty acid ratios GLA/LA, DGLA/GLA, and ARA/DGLA (32, 33, 35). **Table 3** shows these baseline (unsupplemented) results for the primary FAs and FA ratios examined in this study; boxplots are shown for both FAs and FA ratios at baseline in **Supplemental Figure 1**. Importantly, ARA showed highly significant ($p=1.59x\ 10^{-5}$) baseline differences between genotype groups, with each copy of the T allele corresponding to a decrease of 1.39 percentage points (95% CI: (0.95,1.84)) such that the average values of ARA were 8.13% for those with the GG genotype but only 5.39% for TT individuals (i.e., representing the inefficient haplotype). The ARA/DGLA ratio, reflecting FADS1 activity, was also highly genotype-dependent (GG, 5.67> GT, 4.20> TT, 3.11; $p=1.76 \times 10^{-11}$).

Effect of Oil Supplementation and rs174537 Genotype on Serum Fatty Acids

In this crossover study, each participant consumed both the control oil (SO) and the experimental oil (BO), albeit in an arm-dependent order. The washout period of 8

weeks between the oil consumption periods was deemed successful, as we did not observe differences in serum levels of GLA, the principle fatty acid differing between the two oils (**Table 1**), between the first (V2) and the second (V5) baselines for all subjects and genotypes (diff=0.12, 95% CI: (-0.016, 0.04); p=0.39, paired t-test). LA and DGLA also had negligible differences between arms, and those results are presented in **Supplemental Table 1**.

The time courses of serum levels of n-6 PUFAs and LC-PUFAs in response to BO and SO supplementation are shown in **Figure 3**. There were clear differences by genotype in the response to BO supplementation, but no changes with SO. Based on the fatty acid composition of the supplemental oils (**Table 1**), both oils would be expected to enter the n-6 LC-PUFA biosynthetic pathway (**Figure 1**). As expected, for all genotypes, GLA levels markedly increased within 2 weeks after BO consumption but not after SO. Mean values increased several-fold after four weeks of BO consumption. Comparing genotypes, the “inefficient” TT genotype had the lowest mean value at baseline but also increased the most over the study period (**Figure 3**). Thus, while the week 4 differences between genotypes were not significantly different from each other (p=0.81, test of Week 4 x rs174537 interaction for BO), GLA increased ~3-fold in those with the GG genotype vs ~6-fold in TT individuals. Given the trajectories in **Figure 3**, a longer supplementation period may have resulted in larger genotypic differences. DGLA, the elongation product of GLA, also increased significantly during BO consumption, but not during SO consumption. Baseline DGLA levels were 1-2% and increased only slightly with each copy of the T allele (diff=0.19, 95% CI: (0.075, 0.30); Table 3). Values after four weeks, however, were strongly connected to genotype, such

that TT subjects had dramatically higher values than GG subjects (4.1% vs 2.2% for TT and GG, respectively). Although less striking, levels of ARA, the FADS1 desaturation product of DGLA, significantly increased by ~18% ($p=7.39 \times 10^{-11}$; **Table 4**) with BO consumption for all genotypes. This elevation was seen in GT and GG individuals after just 2 weeks of supplementation, while increased ARA levels in the TT individuals were only evident after 4 weeks of BO consumption (see **Supplemental Table 2**). SO supplementation resulted in no changes in ARA levels compared to baseline ($p= 0.061$).

Table 4 compares the impact of oil supplementation on major n-6 and n-3 PUFAs and LC-PUFAs over the course of the study period. For each fatty acid, we fit a linear mixed-effects model to the longitudinal data and estimated the changes in FA over the four weeks of supplementation. Interestingly, both LA (-5%) and EPA (-16%) levels also showed small, but statistically significant decreases with BO consumption. In contrast, the levels of GLA, DGLA and ARA increased nearly 400%, 200% and 18%, respectively. No changes in other n-3 LC-PUFAs (DPA and DHA) levels were observed (see **Supplemental Figure 2**) with the consumption of either botanical oil.

During BO consumption, both GLA and DGLA levels varied significantly with genotype, such that the accumulation of circulating GLA and DGLA increased dramatically with each additional copy of the T allele. Relatively small baseline differences grew over time such that TT individuals had nearly double the levels of DGLA at the end of the four weeks of supplementation period. Other co-variants (sex, age, oil supplementation sequence) in this model did not impact fatty acid levels. These data clearly reveal a gene-diet interaction for BO.

The increase in serum ARA with BO consumption appear to be modest

compared to those for GLA and DGLA and it appears to not vary greatly by genotype. However, it is important to recognize that GLA and DGLA are low abundance fatty acids in the circulation with values normally ranging from <1% of total circulating fatty acids for GLA and <3% for DGLA. In contrast, circulating levels of ARA range from 5-10% of total fatty acids. **Supplemental Table 2** shows an analysis of the circulating ARA levels (expressed as % of total fatty acids) in this cohort at baseline and after 4 weeks of oil supplementation. As we and others have previously observed, baseline levels of ARA are genotype-dependent (GG>GT>TT) and highly associated with rs174537 (33, 35). Importantly, as illustrated in **Figure 3**, addition of GLA-containing BO increased ARA levels in all individuals but it reached the highest levels in individuals with rs174537 G alleles. Even after 4 weeks of BO supplementation, TT individuals reached an average of 6.41% ARA (95% CI: (5.55, 7.27), **Figure 3**), which was still lower than GG individuals at baseline with an average of 8.13% (95% CI: (7.48, 8.83), **Table 3**). We did not observe any similar changes with SO supplementation.

We next applied this analysis approach to the fatty acid ratios that serve as surrogates for enzymatic activity for the steps in the PUFA metabolic pathway shown in **Figure 1**: specifically, GLA/LA (FADS2), DGLA/GLA (ELOVL5*)* and ARA/DGLA (FADS1). These results are shown graphically in **Supplemental Figure 3**. The apparent increase in FADS2 and decrease in ELOLV5 activities were likely driven by the elevated GLA levels during BO supplementation. With BO consumption, the ARA/DGLA ratio representing the FADS1 step, which is two steps removed from the consumed n-6 PUFA (GLA), decreased for all three genotypes, but showed the largest decrease for TT

individuals. In contrast, SO consumption resulted in no change in the ARA/DGLA ratio. This provides further evidence for an allelic effect and a gene-diet interaction.

Effect of Botanical Oil Supplementation on Cardiometabolic and Inflammatory Biomarkers

The impact of oil supplementation (4-weeks consumption of each oil) on cardiometabolic and inflammatory biomarkers was evaluated by comparing baseline and week 4 values of serum lipids, high sensitivity C-reactive protein (hsCRP) and blood glucose (**Table 5**). Neither SO nor BO consumption impacted other measured endpoints in any genotype subgroup over the 4-week supplementation period.

DISCUSSION

Early clinical studies suggested that GLA-enriched botanical oils positively impact the symptoms of several chronic inflammatory diseases, but more recent reviews and meta-analyses have questioned their effectiveness (2-15). A number of important issues may be responsible for much of this heterogeneity including low subject numbers, less than ideal clinical design, GLA sources and administration, and disease states examined (15, 59). However, there is also a growing body of evidence that suggests there may be important relationships between genetic variation in the human host and the effectiveness of bioactive components in natural products (60). With regard to GLA-containing botanical oils, there is evidence that evolutionary-driven, ancestry-based, *FADS* gene-dietary PUFA interactions may impact the balance of glycerolipid substrates

available for anti-and pro-inflammatory eicosanoid biosynthesis (40, 60). Numerous cross-sectional studies point to *FADS1* encoded ∆-5 desaturase efficiency in impacting levels of DGLA, ARA, and importantly the ratio of ARA to DGLA. In fact, Geiger and colleagues pointed out that the effect between *FADS1* variants and the ratio of ARA- to DGLA-containing phosphatidylcholine species is so strong that "if the molecular function of *FADS1* had not been already known, the association between the SNP and the different glycerophospholipid concentrations per se would have allowed (one) to deduce its enzymatic activity of inserting a fourth double bond." (34).

Given this evidence, a critical next step was to complete a prospective clinical trial to determine the impact of *FADS1* variation on the metabolic flux of GLA entry into the LC-PUFA biosynthetic pathway and its subsequent conversion into DGLA and ARA products. The current trial did so, but providing a dietary source of LA from SO and LA+GLA from BO to non-Hispanic White individuals stratified by the *FADS* genotype at rs174537. Overall, there was little difference in circulating fatty acids or cardiometabolic/ inflammatory biomarker levels during SO supplementation for any of the genotype subgroups. This is perhaps not surprising given that the current Western diet contains very high levels of LA (12-15g per day (61)). The addition of ~35% (~5g/day) more LA content in the form of a supplement may not have significant effects over the background diet. This is in contrast to a recent study that provided 17-28g of LA over the background LA levels in the diets. In that study, the *FADS1* genotype did modify metabolic responses and ARA levels in response to high concentrations of LA (62).

In contrast after BO supplementation, the accumulation of DGLA and ARA, and possibly GLA, was genotype dependent. The amount of GLA in the BO supplements

was substantial compared with typical participant diets, and GLA levels increased quickly in all three genotypes (**Figure 3C**). In agreement with our central research hypothesis, accumulation of circulating DGLA differed dramatically by genotype, and resulted in marked alterations in the ratios of ARA to DGLA. Early biochemical studies of this pathway suggested that dietary GLA is rapidly elongated via an *ELOVL5* elongase activity to DGLA, and the *FADS1* (Δ-5 desaturase) step represents the major "bottleneck" in the conversion of GLA to ARA (30, 63). We postulate that after BO causes GLA to rise in all subjects, it is converted to DGLA via ELOVL5 and/or ELOVL2, but then reaches the rate-limiting FADS1 step. Then, individuals with the GG genotype efficiently metabolize DGLA -> ARA, while DGLA and GLA accumulate in the TT individuals due to their less efficient FADS1 metabolism. In general, the data from this prospective clinical trial support this hypothesis but also show that, when examining the metabolic flux through the pathway, *FADS1* variation leads to differential accumulation of DGLA, and possibly also GLA.

A limitation of the current study is that we did not have the capacity to measure cyclooxygenase or lipoxygenase metabolites of DGLA and ARA to determine if their quantities were associated with levels and ratios of their precursors. This is difficult in human *in vivo* studies especially with DGLA metabolites as eicosanoids are locally-acting mediators that are typically rapidly metabolized. DGLA has the capacity to be metabolized by cyclooxygenase to 1-series PGs, particularly PGE_1, and by 15-lipoxygenase into 15-HETrE, but these are cell/tissue dependent metabolic steps. We were not able to detect circulating levels of DGLA metabolites in human blood utilizing LC-MS/MS (data not shown), but this is not surprising given their rapid metabolism and

cell/tissue specific biosynthesis (25, 64). Our previous study focusing on eicosanoids produced by stimulated whole blood demonstrated associations between genotype at rs174537 and eicosanoids, including leukotriene B_4 and 5-HETE. This indicates that rs174537 not only impacts the synthesis of ARA precursors but also that of ARA bioactive metabolites (65). Given the biological effects of DGLA metabolites and their function in a wide variety of disorders/diseases (66-69), it is likely that quantities of these metabolites and their balance to ARA metabolites plays a key role in human health and disease.

Like many natural products that contain potential bioactives, there has been large heterogeneity in the results derived from clinical trials aimed at interrogating GLA-containing oils. Clearly, genetic variations within the human host have a great capacity to impact the outcomes in natural product supplementation studies. The current study clearly suggests that gene-diet interactions exist in the context of PUFA metabolism. Thus, understanding that for individuals with a variant(s) resulting in the efficient PUFA metabolizing phenotype, GLA supplementation could lead to elevated ARA precursors, eicosanoids, and undesirable clinical outcomes. The opposite would be true for individuals with variant(s) resulting in the inefficient PUFA metabolizing phenotype. Our results suggest that the balance of DGLA and ARA precursors, both up- or down-stream of the FADS1 step, can contribute to the supplement's impact in a genotype subgroup. In conclusion, the current results clearly suggest that personalized- and population-based approaches may be necessary to optimize the design of future clinical studies with GLA-containing oils.

ACKNOWLEDGEMENTS

The authors acknowledge and thank the individuals who gave generously of their time to participate in the study. The authors also thank the following people who provided important assistance in conducting the study. Keri Marshall and Nordic Naturals were instrumental in helping to provide the high-quality encapsulated oil used for this study. Tim Morgan, Ph.D. performed the randomization of participants to study arms. Brian Strittmatter, PharmD blinded the study oil products before they were dispensed to participants. Edward Kirby provided valuable assistance with fatty acid analyses. Kirsten Lake provided important editorial assistance during the preparation of the manuscript.

Authors' contributions: FHC and RAM designed the study and protocols, FHC oversaw the conduct of the study; SS and FHC wrote the manuscript; TLM, PI and MLB were study coordinators; SS analyzed samples; SS performed data analyses; IR, BH, LJ performed the statistical analyses; SS oversaw the acquisition of the encapsulated oils, study product monitoring and quality control; MCS oversaw data quality control and edited the manuscript.

All authors read and approved the final version of the manuscript.

REFERENCES

1. Sergeant S, Rahbar E, Chilton FH. Gamma-linolenic acid, Dihommo-gamma linolenic, Eicosanoids and Inflammatory Processes. *Eur J Pharmacol* 2016;785:77-86. doi: 10.1016/j.ejphar.2016.04.020.

2. Andreassi M, Forleo P, Di Lorio A, Masci S, Abate G, Amerio P. Efficacy of gamma-linolenic acid in the treatment of patients with atopic dermatitis. *J Int Med Res* 1997;25(5):266-74. doi: 10.1177/030006059702500504.

3. Foolad N, Brezinski EA, Chase EP, Armstrong AW. Effect of nutrient supplementation on atopic dermatitis in children: a systematic review of probiotics, prebiotics, formula, and fatty acids. *JAMA Dermatol* 2013;149(3):350-5.

4. Kunkel SL, Ogawa H, Ward PA, Zurier RB. Suppression of chronic inflammation by evening primrose oil. Prog Lipid Res 1981;20:885-8.

5. Leventhal LJ, Boyce EG, Zurier RB. Treatment of rheumatoid arthritis with gammalinolenic acid. *Ann Intern Med* 1993;119(9):867-73. doi: 10.7326/0003-4819-119-9-199311010-00001.

6. Leventhal LJ, Boyce EG, Zurier RB. Treatment of rheumatoid arthritis with blackcurrant seed oil. *Br J Rheumatol* 1994;33(9):847-52.

7. Lovell CR, Burton JL, Horrobin DF. Treatment of atopic eczema with evening primrose oil. *Lancet* 1981;1(8214):278. doi: 10.1016/s0140-6736(81)92119-x.

8. Morse PF, Horrobin DF, Manku MS, Stewart JC, Allen R, Littlewood S, Wright S, Burton J, Gould DJ, Holt PJ, et al. Meta-analysis of placebo-controlled studies of the efficacy of Epogam in the treatment of atopic eczema. Relationship between plasma essential fatty acid changes and clinical response. *Br J Dermatol* 1989;121(1):75-90. doi: 10.1111/j.1365-2133.1989.tb01403.x.

9. Tate G, Mandell BF, Laposata M, Ohliger D, Baker DG, Schumacher HR, Zurier RB. Suppression of acute and chronic inflammation by dietary gamma linolenic acid. *J Rheumatol* 1989;16(6):729-34.

10. Zurier RB, Rossetti RG, Jacobson EW, DeMarco DM, Liu NY, Temming JE, White BM, Laposata M. gamma-Linolenic acid treatment of rheumatoid arthritis. A randomized, placebo-controlled trial. *Arthritis Rheum* 1996;39(11):1808-17.

11. Bamford JT, Ray S, Musekiwa A, van Gool C, Humphreys R, Ernst E. Oral evening primrose oil and borage oil for eczema. *Cochrane Database Syst Rev* 2013(4):Cd004416. doi: 10.1002/14651858.CD004416.pub2.

12. Belch JJ, Hill A. Evening primrose oil and borage oil in rheumatologic conditions. *Am J Clin Nutr* 2000;71(1 Suppl):352s-6s. doi: 10.1093/ajcn/71.1.352s.

13. Kitz R, Rose MA, Schonborn H, Zielen S, Bohles HJ. Impact of early dietary gamma-linolenic acid supplementation on atopic eczema in infancy. *Pediatr Allergy Immunol* 2006;17(2):112-7. doi: 10.1111/j.1399-3038.2005.00369.x.

14. Macfarlane GJ, El-Metwally A, De Silva V, Ernst E, Dowds GL, Moots RJ. Evidence for the efficacy of complementary and alternative medicines in the management of rheumatoid arthritis: a systematic review. *Rheumatology* (Oxford) 2011;50(9):1672-83. doi: 10.1093/rheumatology/ker119.

15. van Gool CJ, Zeegers MP, Thijs C. Oral essential fatty acid supplementation in atopic dermatitis-a meta-analysis of placebo-controlled trials. *Br J Dermatol* 2004;150(4):728-40. doi: 10.1111/j.0007-0963.2004.05851.x.

16. de Bravo MM, De Tomas ME, Mercuri O. Metabolism of gammalinolenic acid by human blood platelet microsomes. *Biochem Int* 1985;10(6):889-96.

17. Chapkin RS, Ziboh VA, Marcelo CL, Voorhees JJ. Metabolism of essential fatty acids by human epidermal enzyme preparations: evidence of chain elongation. J *Lipid Res* 1986;27(9):945-54.

18. Chilton L, Surette ME, Swan DD, Fonteh AN, Johnson MM, Chilton FH. Metabolism of gammalinolenic acid in human neutrophils. *J Immunol* 1996;156(8):2941-7.

19. Wang X, Lin H, Gu Y. Multiple roles of dihomo-gamma-linolenic acid against proliferation diseases. *Lipids Health Dis* 2012;11:25. doi: 10.1186/1476-511x-11-25.

20. Tabolacci C, Lentini A, Provenzano B, Gismondi A, Rossi S, Beninati S. Similar antineoplastic effects of nimesulide, a selective COX-2 inhibitor, and prostaglandin E1 on B16-F10 murine melanoma cells. *Melanoma Res* 2010;20(4):273-9. doi: 10.1097/CMR.0b013e328339d8ac.

21. Williams CD, Whitley BM, Hoyo C, Grant DJ, Iraggi JD, Newman KA, Gerber L, Taylor LA, McKeever MG, Freedland SJ. A high ratio of dietary n-6/n-3 polyunsaturated fatty acids is associated with increased risk of prostate cancer. *Nutr Res* 2011;31(1):1-8. doi: 10.1016/j.nutres.2011.01.002.

22. Hofmanova J, Vaculova A, Kozubik A. Polyunsaturated fatty acids sensitize human colon adenocarcinoma HT-29 cells to death receptor-mediated apoptosis. *Cancer Lett* 2005;218(1):33-41. doi: 10.1016/j.canlet.2004.07.038.

23. Skuladottir GV, Heidarsdottir R, Arnar DO, Torfason B, Edvardsson V, Gottskalksson G, Palsson R, Indridason OS. Plasma n-3 and n-6 fatty acids and

the incidence of atrial fibrillation following coronary artery bypass graft surgery. *Eur J Clin Invest* 2011;41(9):995-1003. doi: 10.1111/j.1365-2362.2011.02497.x.

24. Gallagher H, Williams JO, Ferekidis N, Ismail A, Chan YH, Michael DR, Guschina IA, Tyrrell VJ, O'Donnell VB, Harwood JL, et al. Dihomo-gamma-linolenic acid inhibits several key cellular processes associated with atherosclerosis. *Biochim Biophys Acta Mol Basis Dis* 2019;1865(9):2538-50. doi: 10.1016/j.bbadis.2019.06.011.
25. Borgeat P, Hamberg M, Samuelsson B. Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. *J Biol Chem* 1976;251(24):7816-20.
26. Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. *Annu Rev Biochem* 1986;55:69-102. doi: 10.1146/annurev.bi.55.070186.000441.
27. Iversen L, Fogh K, Kragballe K. Effect of dihomogammalinolenic acid and its 15-lipoxygenase metabolite on eicosanoid metabolism by human mononuclear leukocytes in vitro: selective inhibition of the 5-lipoxygenase pathway. *Arch Dermatol Res* 1992;284(4):222-6.
28. Chapkin RS, Miller CC, Somers SD, Erickson KL. Ability of 15-hydroxyeicosatrienoic acid (15-OH-20:3) to modulate macrophage arachidonic acid metabolism. *Biochem Biophys Res Commun* 1988;153(2):799-804. doi: 10.1016/s0006-291x(88)81166-5.

29. Arm JP, Boyce JA, Wang L, Chhay H, Zahid M, Patil V, Govindarajulu U, Ivester P, Weaver KL, Sergeant S, et al. Impact of botanical oils on polyunsaturated fatty acid metabolism and leukotriene generation in mild asthmatics. *Lipids Health Dis* 2013;12:141. doi: 10.1186/1476-511x-12-141.

30. Johnson MM, Swan DD, Surette ME, Stegner J, Chilton T, Fonteh AN, Chilton FH. Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. *J Nutr* 1997;127(8):1435-44. doi: 10.1093/jn/127.8.1435.

31. Martin SA, Brash AR, Murphy RC. The discovery and early structural studies of arachidonic acid. *J Lipid Res* 2016;57(7):1126-32. doi: 10.1194/jlr.R068072.

32. Mathias RA, Vergara C, Gao L, Rafaels N, Hand T, Campbell M, Bickel C, Ivester P, Sergeant S, Barnes KC, et al. FADS genetic variants and omega-6 polyunsaturated fatty acid metabolism in a homogeneous island population. *J Lipid Res* 2010;51(9):2766-74. doi: 10.1194/jlr.M008359.

33. Mathias RA, Sergeant S, Ruczinski I, Torgerson DG, Hugenschmidt CE, Kubala M, Vaidya D, Suktitipat B, Ziegler JT, Ivester P, et al. The impact of FADS genetic variants on omega6 polyunsaturated fatty acid metabolism in African Americans. *BMC Genet* 2011;12:50. doi: 10.1186/1471-2156-12-50.

34. Gieger C, Geistlinger L, Altmaier E, Hrabe de Angelis M, Kronenberg F, Meitinger T, Mewes HW, Wichmann HE, Weinberger KM, Adamski J, et al. Genetics meets metabolomics: a genome-wide association study of metabolite profiles in human serum. *PLoS Genet* 2008;4(11):e1000282. doi: 10.1371/journal.pgen.1000282.

35. Sergeant S, Hugenschmidt CE, Rudock ME, Ziegler JT, Ivester P, Ainsworth HC, Vaidya D, Case LD, Langefeld CD, Freedman BI, et al. Differences in arachidonic acid levels and fatty acid desaturase (FADS) gene variants in African Americans and European Americans with diabetes or the metabolic syndrome. *Br J Nutr* 2012;107(4):547-55. doi: 10.1017/s0007114511003230.

36. Ameur A, Enroth S, Johansson A, Zaboli G, Igl W, Johansson AC, Rivas MA, Daly MJ, Schmitz G, Hicks AA, et al. Genetic adaptation of fatty-acid metabolism: a human-specific haplotype increasing the biosynthesis of long-chain omega-3 and omega-6 fatty acids. *Am J Hum Genet* 2012;90(5):809-20. doi: 10.1016/j.ajhg.2012.03.014.

37. Mathias RA, Fu W, Akey JM, Ainsworth HC, Torgerson DG, Ruczinski I, Sergeant S, Barnes KC, Chilton FH. Adaptive evolution of the FADS gene cluster within Africa. *PLoS One* 2012;7(9):e44926. doi: 10.1371/journal.pone.0044926.

38. Fumagalli M, Moltke I, Grarup N, Racimo F, Bjerregaard P, Jorgensen ME, Korneliussen TS, Gerbault P, Skotte L, Linneberg A, et al. Greenlandic Inuit show genetic signatures of diet and climate adaptation. *Science* 2015;349(6254):1343-7. doi: 10.1126/science.aab2319.

39. Amorim CE, Nunes K, Meyer D, Comas D, Bortolini MC, Salzano FM, Hunemeier T. Genetic signature of natural selection in first Americans. *Proc Natl Acad Sci USA* 2017;114(9):2195-9. doi: 10.1073/pnas.1620541114.

40. Harris DN, Ruczinski I, Yanek LR, Becker LC, Becker DM, Guio H, Cui T, Chilton FH, Mathias RA, O'Connor TD. Evolution of Hominin Polyunsaturated Fatty Acid

Metabolism: From Africa to the New World. *Genome Biol Evol* 2019;11(5):1417-30. doi: 10.1093/gbe/evz071.

41. Mathieson S, Mathieson I. FADS1 and the Timing of Human Adaptation to Agriculture. *Mol Biol Evol* 2018;35(12):2957-70. doi: 10.1093/molbev/msy180.
42. Mathieson I, Lazaridis I, Rohland N, Mallick S, Patterson N, Roodenberg SA, Harney E, Stewardson K, Fernandes D, Novak M, et al. Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* 2015;528(7583):499-503. doi: 10.1038/nature16152.
43. Li P, Zhao J, Kothapalli KSD, Li X, Li H, Han Y, Mi S, Zhao W, Li Q, Zhang H, et al. A regulatory insertion-deletion polymorphism in the FADS gene cluster influences PUFA and lipid profiles among Chinese adults: a population-based study. *Am J Clin Nutr* 2018;107(6):867-75. doi: 10.1093/ajcn/nqy063.
44. Kothapalli KSD, Ye, Kaixiong, Gadgil MS, Carlson SE, O'Brien KO, Zhang JY, Park HG, Ojukwu K, Zou J, Hyon SS, et al. Positive Selection on a Regulatory Insertion–Deletion Polymorphism in FADS2 Influences Apparent Endogenous Synthesis of Arachidonic Acid. *Mol Biol Evol* 2016;33(7):1726-39. doi: 10.1093/molbev/msw049.
45. Brenna JT, Plourde M, Stark KD, Jones PJ, Lin Y-H. Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *Am J Clin Nutr* 2018;108(2):211-27. doi: 10.1093/ajcn/nqy089.
46. Sorkin BC, Kuszak AJ, Williamson JS, Hopp DC, Betz JM. The Challenge of Reproducibility and Accuracy in Nutrition Research: Resources and Pitfalls. *Adv Nutr* 2016;7(2):383-9. doi: 10.3945/an.115.010595.

47. Kuszak AJ, Hopp DC, Williamson JS, Betz JM, Sorkin BC. Approaches by the US National Institutes of Health to support rigorous scientific research on dietary supplements and natural products. *Drug Test Anal* 2016;8(3-4):413-7. doi: 10.1002/dta.1931.

48. Sorkin B, Kuszak A, Pauli G, Bloss G, Barrett B, Ferruzzi M, Fukagawa N, Kiely M, Lakens D, Meltzer D, et al. Enhancing Natural Product Clinical Trials (P13-037-19). *Curr Dev Nutr* 2019;3(Suppl 1):nzz036.P13-7-19. doi: 10.1093/cdn/nzz036.P13-037-19.

49. Metcalfe LD, Schmitz AA, Pelka JR. Rapid Preparation of Fatty Acid Esters from Lipids for Gas Chromatographic Analysis. *Anal Chem* 1966;38(3):514-5. doi: 10.1021/ac60235a044.

50. Sergeant S, Ruczinski I, Ivester P, Lee TC, Morgan TM, Nicklas BJ, Mathias RA, Chilton FH. Impact of methods used to express levels of circulating fatty acids on the degree and direction of associations with blood lipids in humans. *Br J Nutr* 2016;115(2):251-61. doi: 10.1017/s0007114515004341.

51. Weaver KL, Ivester P, Seeds M, Case LD, Arm JP, Chilton FH. Effect of dietary fatty acids on inflammatory gene expression in healthy humans. *J Biol Chem* 2009;284(23):15400-7. doi: 10.1074/jbc.M109.004861.

52. Council of Europe. Directorate for the Quality of M. 2.5 Assays. Edtion ed. *European Pharmacopoeia*. Strasbourg: Directorate for the Quality of Medicines, Council of Europe, 2010:137-8.

53. Talbot G. The Stability and Shelf Life of Fats and Oils. Edtion ed. In: Subramaniam P, ed. *The Stability and Shelf Life of Food* (Second Edition): Woodhead Publishing, 2016:461-503.

54. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2018. Available online at https://www.R-project.org/.

55. Aitchison J. The statistical analysis of compositional data. London; Chapman and Hall, 1986.

56. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. nlme: Linear and Nonlinear Mixed Effects Models. 2019. https://CRAN.R-project.org/package=nlme.

57. Amrhein V, Greenland S, McShane B. Scientists rise up against statistical significance. *Nature* 2019;567(7748):305-7. doi: 10.1038/d41586-019-00857-9.

58. Rennie D. CONSORT revised--improving the reporting of randomized trials. *JAMA* 2001;285(15):2006-7. doi: 10.1001/jama.285.15.2006.

59. Foster RH, Hardy G, Alany RG. Borage oil in the treatment of atopic dermatitis. *Nutrition* 2010;26(7-8):708-18. doi: 10.1016/j.nut.2009.10.014.

60. Chilton FH, Dutta R, Reynolds LM, Sergeant S, Mathias RA, Seeds MC. Precision Nutrition and Omega-3 Polyunsaturated Fatty Acids: A Case for Personalized Supplementation Approaches for the Prevention and Management of Human Diseases. *Nutrients* 2017;9(11). doi: 10.3390/nu9111165.

61. Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *Am J Clin Nutr* 2011;93(5):950-62. doi: 10.3945/ajcn.110.006643.

62. Lankinen MA, Fauland A, Shimizu BI, Agren J, Wheelock CE, Laakso M, Schwab U, Pihlajamaki J. Inflammatory response to dietary linoleic acid depends on FADS1 genotype. *Am J Clin Nutr* 2019;109(1):165-75. doi: 10.1093/ajcn/nqy287.

63. Barham JB, Edens MB, Fonteh AN, Johnson MM, Easter L, Chilton FH. Addition of eicosapentaenoic acid to gamma-linolenic acid-supplemented diets prevents serum arachidonic acid accumulation in humans. *J Nutr* 2000;130(8):1925-31. doi: 10.1093/jn/130.8.1925.

64. Levin G, Duffin KL, Obukowicz MG, Hummert SL, Fujiwara H, Needleman P, Raz A. Differential metabolism of dihomo-gamma-linolenic acid and arachidonic acid by cyclo-oxygenase-1 and cyclo-oxygenase-2: implications for cellular synthesis of prostaglandin E1 and prostaglandin E2. *Biochem J* 2002;365(Pt 2):489-96. doi: 10.1042/bj20011798.

65. Hester AG, Murphy RC, Uhlson CJ, Ivester P, Lee TC, Sergeant S, Miller LR, Howard TD, Mathias RA, Chilton FH. Relationship between a common variant in the fatty acid desaturase (*FADS*) cluster and eicosanoid generation in humans. *J Biol Chem* 2014;289(32):22482-9. doi: 10.1074/jbc.M114.579557.

66. Ye Z, Lu H, Guo W, Dai W, Li H, Yang H, Li L. The effect of alprostadil on preventing contrast-induced nephropathy for percutaneous coronary intervention in diabetic patients: A systematic review and meta-analysis. *Medicine* (Baltimore) 2016;95(46):e5306. doi: 10.1097/md.0000000000005306.

67. Vietto V, Franco JV, Saenz V, Cytryn D, Chas J, Ciapponi A. Prostanoids for critical limb ischaemia. *Cochrane Database Syst Rev* 2018;1:Cd006544. doi: 10.1002/14651858.CD006544.pub3.

68. McLaughlin J, Devoe LD. Current Status of Prostaglandins for Cervical Ripening. *J Reprod Med* 2017;62(5-6):221-8.

69. Moncada I, Martinez-Salamanca J, Ruiz-Castane E, Romero J. Combination therapy for erectile dysfunction involving a PDE5 inhibitor and alprostadil. *Int J Impot Res* 2018;30(5):203-8. doi: 10.1038/s41443-018-0046-2.

TABLES

Table 1. Fatty Acid Profile of Encapsulated Study Oils.

		Fatty Acid Profile Encapsulated Oils (area %)		PUFAs provided by Supplementation (g/day)	
Common name	**Fatty Acid**	**Soybean**	**Borage**	**Soybean**	**Borage**
palmitic	C16:0	10.5	10.2	1.09	1.13
stearic	C18:0	4.5	4.0	0.59	0.44
oleic	C18:1 n-9c	24.4	15.7	2.50	1.73
vaccenic	C18:1 n-7c	1.5	0.6	0.16	0.06
linoleic (LA)	C18:2 n-6c	49.7	37.3	5.27	4.11
γ-linolenic (GLA)	C18:3 n-6	0	23.0	0	2.54
a-linolenic (ALA)	C18:3 n-3	6.2	0.2	0.68	0.02
stearidonic (SDA)	C18:4 n-3	0	0.2	0	0.02
arachidic	C20:0	0.4	0.2	0.03	0.03
gondoic	C20:1 n-9	0.3	4.2	0.02	0.46
eicosadienoic	C20:2 n-6	0	0.2	0	0.02
behenic	C22:0	0.4	0.1	0.03	0.02
euricic	C22:1 n-9	0	2.6	0	0.29
	C24:1 n-9	0	1.3	0	0.15
	others	0.1	0.1	0	0.01
	total	98.0	99.9	10.37	11.02

The fatty acid profile of the control (soybean) and experimental (borage) oils are derived from in-house quality control analyses are in agreement with that stated on vendor supplied Certificates of Analysis. The dose of fatty acids was provided in the 10 capsules consumed daily by participants.

Table 2. Baseline Demographics of the Study Cohort

	GG	GT	TT
Female, %	92	66.7	55.6
n	25	30	9
age	39.7 (12.7)	40.6 (11.7)	41.3 (9.9)
Systolic BP	110.1 (11.46)	111.9 (13.0)	113.1 (11.0)
Diastolic BP	66.0(7.04)	69.3 (9.2)	71.73 (9.9)
Resting HR	66.6 (9.39)	67.0 (12.7)	70.3 (11.17)
Weight, kg	65.0 (9.7)	73.2 (11.0)	71.9 (13.2)
% Body Fat	29.6 (7.5)	28.21 (9.4)	25.8 (9.1)
BMI	23.2 (2.8)	24.9 (2.9)	24.4 (2.7)
Waist, cm	69.7 (6.3)	78.2 (7.9)	76.3 (12.1)
Hip, cm	94.8 (7.8)	98.0 (6.4)	96.2 (6.4)
Waist/Hip ratio	0.74 (0.04)	0.80 (0.07)	0.79 (0.08)

The values shown are the mean (sd) at the V2 baseline before oil supplementation for the 64 participants that began the intervention.

Table 3. Baseline FA and FA ratio values by genotype.

FA or Ratio	rs174537 Genotype	Baseline		
		Mean	95% CI	p-value
LA	GG	35.13	(33.79, 36.50)	0.76
	GT	35.34	(34.40, 36.29)	
	TT	35.55	(33.68, 37.46)	
GLA	GG	0.42	(0.35, 0.50)	**0.0002**
	GT	0.30	(0.26, 0.33)	
	TT	0.21	(0.16, 0.27)	
DGLA	GG	1.44	(1.30, 1.59)	0.07
	GT	1.58	(1.47, 1.69)	
	TT	1.73	(1.51, 1.98)	
ARA	GG	8.13	(7.48, 8.83)	**1.59×10^{-5}**
	GT	6.63	(6.25, 7.02)	
	TT	5.39	(4.78, 6.07)	
EPA	GG	0.48	(0.40, 0.57)	0.0024
	GT	0.36	(0.32, 0.41)	
	TT	0.28	(0.22, 0.35)	
DPA	GG	0.41	(0.36, 0.47)	0.07
	GT	0.36	(0.33, 0.40)	
	TT	0.32	(0.26, 0.38)	
DHA	GG	1.29	(1.13, 1.48)	0.99
	GT	1.29	(1.18, 1.42)	
	TT	1.29	(1.07, 1.56)	
GLA/LA	GG	0.012	(0.010, 0.014)	**4.09×10^{-5}**
	GT	0.008	(0.007, 0.010)	
	TT	0.006	(0.005, 0.008)	
DGLA/GLA	GG	3.46	(2.98, 4.01)	**7.74×10^{-11}**
	GT	5.33	(4.81, 5.90)	
	TT	8.21	(6.69, 10.08)	

ARA/DGLA	GG	5.67	(5.02, 6.39)	**1.76 x 10^{-11}**
	GT	4.20	(3.86, 4.56)	
	TT	3.11	(2.63, 3.67)	

This table shows baseline means and 95% confidence intervals for key FAs and FA ratios. The baseline data for both oil consumption periods (visit 2 and visit 5) was used since the washout period was successful (Supplemental Table 1). FA values are presented as % of total FAs. The p-values are from tests of whether that FA or ratio differed by genotype. The bold values highlight the FAs and ratios that showed significant differences by genotype at baseline.

Table 4. Evidence of a Gene-Diet Interaction for Borage Oil

		Borage Oil				Soybean Oil			
Fatty Acid	**rs174537 Genotype**	***Change in % of total FAs***		***4-week fold-change***		***Change in % of total FAs***		***4-week fold-change***	
		Estimate	*95% CI*	*Estimate*	*95% CI*	*Estimate*	*95% CI*	*Estimate*	*95% CI*
LA	GG	-1.66	(-1.94, -1.37)	0.95	(0.92, 0.99)	1.05	(0.75, 1.36)	1.02	(0.97, 1.07)
	GT	-2.16	(-2.33, -1.97)	0.94	(0.92, 0.96)	1.19	(0.97, 1.41)	1.03	(0.99, 1.06)
	TT	-2.65	(-2.96, -2.31)	0.93	(0.92, 0.97)	1.32	(0.92, 1.75)	1.04	(0.98, 1.11)
GLA	GG	**0.80**	(0.67, 0.97)	**3.01**	(2.42, 3.74)	0.03	(0.02, 0.04)	1.07	(0.97, 1.11)
	GT	**0.96**	(0.85, 1.09)	**4.21**	(3.63, 4.88)	0.00	(0.00, 0.00)	0.99	(0.94, 1.03)
	TT	**1.09**	(0.85, 1.39)	**5.89**	(4.42, 7.86)	-0.02	(-0.01, -0.02)	0.93	(0.88, 0.97)
DGLA	GG	**0.80**	(0.69, 0.91)	**1.57**	(1.38, 1.79)	-0.08	(-0.09, -0.07)	0.94	(0.87, 1.02)
	GT	**1.46**	(1.34, 1.58)	**1.94**	(1.78, 2.12)	-0.10	(-0.10, -0.09)	0.94	(0.89, 1.00)
	TT	**2.38**	(2.03, 2.79)	**2.41**	(2.03, 2.85)	-0.12	(-0.13, -0.11)	0.95	(0.85, 1.05)
ARA	GG	1.38	(1.27, 1.50)	1.19	(1.17, 1.27)	-0.07	(-0.07, -0.07)	1.00	(0.94, 1.07)
	GT	1.12	(1.06, 1.19)	1.18	(1.13, 1.24)	-0.26	(-0.25, -0.27)	0.98	(0.94, 1.02)
	TT	0.90	(0.80, 1.02)	1.18	(1.08, 1.28)	-0.37	(-0.33, -0.41)	0.95	(0.88, 1.04)
EPA	GG	-0.10	(-0.08, -0.12)	0.79	(0.68, 0.92)	-0.01	(-0.01, -0.01)	1.01	(0.88, 1.16)
	GT	-0.05	(-0.05, -0.06)	0.85	(0.77, 0.94)	0.00	(0.00, 0.00)	0.99	(0.90, 1.09)
	TT	-0.02	(-0.01, -0.02)	0.92	(0.75, 1.12)	0.00	(0.00, 0.00)	0.97	(0.80, 1.17)
DPA	GG	-0.03	(-0.03, -0.03)	0.94	(0.87, 1.02)	0.00	(0.00, 0.00)	1.03	(0.97, 1.10)
	GT	-0.01	(-0.01, -0.01)	0.97	(0.92, 1.03)	0.00	(-0.01,0.01)	0.99	(0.95, 1.03)
	TT	0.00	(0.00, 0.00)	1.01	(0.90, 1.12)	-0.01	(-0.01, 0.01)	0.95	(0.88, 1.03)
DHA	GG	-0.06	(-0.07, -0.05)	0.95	(0.88, 1.02)	-0.10	(-0.11, -0.09)	0.92	(0.85, 0.99)
	GT	-0.04	(-0.05, -0.04)	0.96	(0.91, 1.01)	-0.05	(-0.05, -0.04)	0.96	(0.91, 1.01)
	TT	-0.02	(-0.03, -0.02)	0.98	(0.88, 1.08)	0.00	(0.00, 0.00)	1.00	(0.90, 1.10)

This table shows changes from baseline for important n-3 and n-6 PUFA and LC-PUFA over the course of this study for both oils. Differences between week 4 and week 0 FA values and 95% confidence intervals (CIs) were estimated from the mixed-effects models using all subjects (n=64). Fold-change values and CIs were computed for all individuals that completed both supplementation periods and having data for both time points (n=53). Bold highlights the values for GLA and DGLA on BO, the main focus of this study. For GLA, similar increases in % of total FAs were seen across genotypes (an increase of ~1% of total FAs). The fold-change values varied substantially, but these differences were not significant (p=0.0024) at our conservative alpha. In contrast, the increase in DGLA as % of

total FAs varied by the number T alleles (p=0.0002) reflecting the gene-diet interaction. ARA levels varied by genotype at baseline and increased by about 18% in all three genotypes. We did not observe meaningful changes with SO supplementation.

Table 5 Impact of Botanical Oil Supplementation on Cardiometabolic and Inflammatory Biomarkers

		GG		GT		TT	
Serum Analyte	***supplement***	***Week 0***	***Week 4***	***Week 0***	***Week 4***	***Week 0***	***Week 4***
Triglyceride	soybean oil	70.4 (4.3)	70.1 (6.4)	78.1 (4.9)	77.4 (5.4)	94.5 (27.2)	90.6 (16.5)
	borage oil	63.9 (3.1)	70.5 (6.2)	78.2 (4.5)	85.5 (9.4)	91.9 (15.7)	76.4 (12.7)
Total cholesterol	soybean oil	167.1 (5.9)	167.3 (8.4)	168.5 (5.7)	165.1 (5.5)	165.0 (5.7)	164.6 (6.6)
	borage oil	165.5 (7.1)	163.3 (6.7)	175.6 (6.2)	173.1 (5.7)	162.4 (3.9)	159.8 (6.4)
HDL	soybean oil	65.4 (3.3)	64.7 (3.4)	58.3 (3.1)	59.3 (3.0)	57.8 (3.1)	61.9 (4.1)
	borage oil	65.9 (3.9)	68.0 (4.0)	60.1 (3.1)	64.7 (3.8)	58.8 (5.9)	61.5 (4.7)
LDL	soybean oil	86.6 (5.2)	89.4 (6.0)	94.5 (5.1)	90.5 (4.6)	88.4 (6.8)	84.5 (6.9)
	borage oil	87.0 (6.0)	81.0 (4.4)	99.8 (5.7)	91.4 (4.3)	85.3 (7.2)	82.9 (7.3)
VLDL	soybean oil	14.0 (0.80)	14.4 (1.3)	15.7 (1.0)	15.4 (1.1)	18.9 (5.5)	18.3 (3.3)
	borage oil	12.7 (0.6)	14.3 (1.3)	15.7 (0.9)	17.1 (1.9)	18.3 (3.2)	15.4 (2.6)
Glucose	soybean oil	88.4 (1.3)	87.0 (1.2)	89.5 (1.2)	89.4 (1.2)	85.5 (1.3)	86.1 (0.7)
	borage oil	88.7 (1.3)	87.6 (1.5)	89.0 (1.0)	91.2 (1.3)	86.1 (2.0)	89.0 (1.8)
hsCRP	soybean oil	0.8 (0.3)	0.5 (0.2)	3.8 (2.0)	1.2 (0.3)	0.9 (0.4)	0.7 (0.2)
	borage oil	0.5 (0.1)	0.6 (0.1)	1.4 (0.3)	1.1 (0.2)	1.8 (1.1)	4.6 (3.8)

Measurements of a standard lipid panel, glucose and C-reactive protein (high sensitivity CRP) were made in fasting serum at all intervention visits. Data (mean, se) are presented for baseline (week 0) and after four weeks of oil supplementation. The 0 and 4 week values were not significantly different (paired t-test, by genotype) for any of the markers measured.

FIGURE LEGENDS

Figure 1. Omega 6 PUFA Pathway

The PUFA metabolic pathway consists of alternating desaturation (FADS) and carbon chain elongation (ELOVL) steps. The fatty acids in the administered in botanical oil supplements (LA and GLA) enter the pathway as shown. The bioactive lipids derived from DGLA and ARA are indicated. The omega 6 (n-6) side of the pathway is shown but the same enzymes utilize omega-3 (n-3) substrates (not shown) in a competitive manner. Bioactive lipid mediators derived from n=6 PUFAs are indicated in dashed boxes.

Figure 2. Recruitment and Study Design

A. The recruitment and retention summary is shown for this nutritional intervention study. **B**. The study design is that of a double-blind, randomized crossover type. Participants were stratified by the *FADS* SNP rs174537 genotype (GG, GT, TT). After the consenting and screening process, eligible subjects (of each genotype) were randomized to begin four weeks consumption of either daily soybean or borage oil capsules, followed by an eight week washout period and then another four weeks consumption of the opposite oil. Participant genotype and oil consumption order were

Figure 3. Time course of serum n-6 PUFA levels after botanical oils supplementation.

This figure shows the changes in circulating n-6 PUFAs upon soybean oil (blue) or borage oil (red) supplementation over the course of the four week study period by rs174537 genotype. Points represent the estimated mean values from the mixed-models and bars represent 95% confidence intervals. For both GLA and DGLA, BO supplementation resulted in dramatic increases from baseline values (both $p<1 \times 10^{-11}$). For GLA, values at week 4 were similar across genotypes ($p=0.80$), while for DGLA they varied significantly (8.05×10^{-7}). GLA and DGLA values at two and four weeks were also significantly different from the corresponding values for SO at weeks 2 and 4 (all $p<1 \times 10^{-6}$). For ARA, changes were modest and initial baseline differences by genotype were carried forward such that the initial ordering (GG > GT > TT) was preserved.