Bioavailability of SPMs in Obese Humans

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Short Title: Immunometabolic Response to SPMs

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PROTOCOL TITLE: Bioavailability of Specialized Pro-Resolving Mediators in Obese Humans

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Protocol Version: 2
Version Date: June 5th 2020

I confirm that I have read this protocol and understand it.

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Date: 6/5/20

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ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation	Definition
14-HDHA	14-hydroxy docosahexaenoic acid
17-HDHA	17-hydroxy docosahexaenoic acid
18-HEPE	18-hydroxy eicosapentaenoic acid
BMI	Body mass index
CyTOF	Cytometry at time of flight
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
HDL	high density lipoprotein
LDL	Low density lipoprotein
NSAIDs	non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
PHI	Personal health information
PUFA	Polyunsaturated fatty acid
SPMs	Specialized Pro-resolving Mediators

PROTOCOL SYNOPSIS

Study Title	Immunometabolic Response to Specialized Pro-Resolving Mediators in Obese Humans
Funder	Departmental funds
Clinical Phase	Pilot study
Study Rationale	Obesity is associated with chronic, low-grade inflammation and impaired immunity, which contributes to the development of metabolic complications such as diabetes, cardiovascular disease, and increased susceptibility to infection due to impaired humoral immunity. The metabolism of immune cells has emerged as a major therapeutic target for improving chronic inflammation. Specialized pro-resolving mediators (SPMs) in particular are lipid-derived metabolites critical in orchestrating the resolution phase of inflammation. However, SPMs are lowered with obesity. The innovation lies in the study of SPMs as therapeutic agents for immune resolution of chronic inflammation that can lead to an improved humoral immune response in the obese. Our first objective is to establish the concentration of SPMs in plasma, serum and peripheral blood mononuclear cells (PBMCs) of obese subjects in response to dietary supplementation of SPMs (aim 1a). We will further determine the immunological fitness of the leukocyte pool, specifically examining B cell antibody production in response to ex-vivo stimulation (aim 1b). Finally, we will correlate metabolic profiles with SPM concentrations (aim 2). Overall, this will help us determine whether SPM supplementation mitigates the impaired humoral immune response of obese subjects and determine the use of SPMs as potential therapeutic agents for the obese.
Study Objective(s)	**Primary Aim 1**: To compare immunological fitness pre- and post-oral SPM administration in the obese. • **Aim 1a**: To quantify SPM concentrations in plasma (pg/mL), serum (pg/mL) and PBMCs before and after SPM supplementation. • **Aim 1b**: To measure antibody responses of B cells in PBMC pool with in vitro stimulation and cytokine production before and after SPM supplementation. **Secondary Aim 2**: To compare metabolic profiles pre- and post-supplementation in response to an oral dose of SPMs in the obese. • **Aim 2a**: To measure fasting insulin (ng/dL) and fasting glucose (mg/dL) before and after intervention. • **Aim 2b**: To correlate pre- and post- metabolic measurements with measured pre- and post- SPM concentrations.

Test Article(s) *(If Applicable)*	The 'SPM Active' dietary supplement will be supplied by Metagenics for intervention.
Study Design	A non-randomized uncontrolled clinical trial with convenience sampling. At total of 24 individuals (12 males & 12 females) will be selected against inclusion/exclusion criteria. Pre- and post- i-ntervention measurements will be collected and analyzed.
Subject Population **key criteria for Inclusion and Exclusion:**	Inclusion Criteria 1. Male and (post-menopausal) female subjects age 50-65 years with BMI 30-40 (obese) who are euglycemic and pre-diabetic Exclusion Criteria 1. Subjects with active autoimmune disease, liver disease, coagulopathy, hypothyroidism, known allergy to fish or shellfish, those taking active asthma medications, anticoagulants, estrogen, testosterone, daily aspirin or NSAIDs, anyone with inability to give informed consent 2. Subjects receiving immunomodulatory or immunosuppressant therapy, with known active malignancy or undergoing treatment for malignancy, and those consuming n-3 PUFA supplements or high consumption of fatty fish (>2 servings per week)
Number Of Subjects	24
Study Duration	The entire study is expected to last 6-9 months. This includes an enrollment period of 3-4 months and data analysis.
Study Phases **Screening** **Study Treatment** **Follow-Up**	(1) Screening: Screening for eligibility will be performed by Dr. Butler and his designated study coordinator at UNC Family Medicine Center during standard pre-operative checkups in a private, closed-door room. Subjects will be asked questions orally during the primary visit to determine if eligibility criteria are met, at which time informed consent and HIPPA authorization will be obtained. (2) Intervention: The intervention is the dietary supplement 'SPM Active' supplied by Metagenics. All subjects will be administered 4 capsules per day for 4 weeks +/- 2-4 days. 12-hour fasting blood and serum will be drawn pre- and post-intervention for each subject by a licensed phlebotomist at the UNC Family Medicine Lab and under the direction of Dr. Butler. (3) Follow-Up: The study coordinator will follow up twice by phone at the end of week 1 and week 3 of supplementation and relay any health concerns to Dr. Butler who may determine if an in-person visit is necessary. Following 4 weeks intervention, patients will discontinue supplementation follow up in a routine manner as designated by their UNC Family Medicine physician.

Efficacy Evaluations	If SPM administration does not lead to increased uptake of SPMs in PBMCs or serum with the first 9 subjects, then we will not conduct CyTOF. Instead, we will spend the funds in conducting a pilot metabololipidomic study of 9 subjects of one sex to determine if 8 weeks of intervention leads to an elevation in the three metabolites of interest. We envision no technical limitations to the study as all the proposed protocols are optimized.
Safety Evaluations	While adverse events are unlikely, if 50% or more of total study participants report intolerable adverse reactions from taking the supplement, we will stop the study.
Sample Size Rationale	The primary aim of the study is designed and powered to measure an increase in 18-HEPE, 17-HDHA, and 14-HDHA in Aim 1. Additional measures in Aim 2 are exploratory, but necessary for future grant applications to demonstrate the feasibility of the approach. At a family-wise error rate of 5% controlling for testing of three metabolites mentioned above, n=9 will provide more than 80% power to detect a 50%-fold change increase in metabolite levels within each subject. We are assuming a similar effect size for both males and females and are doubling the sample size (n=18, 9 per sex) to allow us to subset the data in case we find divergent results between sexes. To account for possible loss-to-follow up, we increased the sample size to 24 (n=12 male + 12 female). Power analysis was conducted assuming a paired t-test on pre- and post-treatment metabolite levels for three pre-specified metabolites. Expected variability of within subject differences in metabolite levels was assessed from a previous dataset and the median standard deviation was calculated across all metabolites.[1] Power analyses were calculated by Dr. Michael Love in the UNC Department of Biostatistics.
Statistical & Analytic Plan	Generally, most datasets will be assessed for normality and homogeneity of variance using the Shapiro-Wilks test and Bartlett test respectively. However, normality will also be assessed via a qqplot of the standardized residuals and we will visualize the distribution of our data with a histogram. In addition, to corroborate the Bartlett test we will check the homogeneity of variance of the residuals (homoscedasticity) using a scale-location plot where the square root of the standardized residuals is plotted. Data that passes both assumptions will be analyzed via a paired two sample Student T-test. Data that fails the assumption of heteroscedasticity will be analyzed using a paired Welch T-test. Another alternative would be to apply a log or square root transformation to achieve heteroscedasticity or approach a Gaussian distribution. However, if the data still fails the assumptions of normality and heteroscedasticity it will be analyzed with a paired Wilcoxon rank sum test. All p-values from these

	analyses will undergo either a Bonferroni or Benjimani-Hochberg p-value adjustment at a 5% false discovery rate.
DATA AND SAFETY MONITORING PLAN	Data will be kept on an encrypted desktop computer protected by multiple passwords in a lab, located on a hall protected by card access in Michael Hooker Research Center, Chapel Hill, NC. De-identified data will be stored on REDCap for use by research team personnel only. Data management will be performed by the PI and the study coordinators. All data acquisition will meet HIPPA and confidentiality standards. The PIs will be responsible for monitoring and reviewing subject data, lab results, study progress, subject safety and confidentiality, and the accuracy and security of the emerging data. They will also be responsible for running the assays and entering all data into redcap for statistical analysis. The co-I, Dr. Butler, will follow-up with patients as necessary; his contact information and that of the study coordinator will also be given to the study subjects.

1 BACKGROUND AND RATIONALE

1.1 Introduction

Chronic inflammation, a central component of obesity, contributes toward the development of numerous metabolic complications such as type 2 diabetes, cardiovascular diseases, and increased susceptibility to infection. The metabolism of immune cells has emerged as a major therapeutic target for improving chronic inflammation, and thereby, whole-body metabolism. Specialized pro-resolving mediators (SPMs) in particular are lipid-derived metabolites that have been shown to orchestrate the resolution of inflammation, regeneration of damaged tissues, and even improve outcomes to infections.[2,3] However, SPMs are lowered with obesity. It is therefore hypothesized that exogenous administration of SPMs to obese humans may have therapeutic value as insulin and glucose sensitizing agents by targeting chronic inflammatory pathways in obesity. Until recently, a major roadblock in the field of obesity and SPMs is that translation of findings from the preclinical level to humans has remained limited. However, this obstacle can now be overcome as the first oral formulation of SPMs has emerged on the market as a dietary supplement. We will lead the field by taking the first steps toward precision administration of SPMs as therapeutic agents for immune resolution of chronic inflammation in the obese. Specifically, we will establish the concentration of SPMs in plasma, serum and peripheral blood mononuclear cells (PBMCs) of obese subjects in response to dietary supplementation with 'SPM Active'. We will further determine the immunological fitness of the leukocyte pool, specifically examining B cell antibody production in response to ex-vivo stimulation. This will determine whether SPM supplementation mitigates the impaired humoral immune response of obese subjects. Overall, this work will help determine the use of SPMs as potential therapeutic agents for the obese.

1.2 Name and Description of Investigational Product or Intervention

The study will provide the dietary supplement 'SPM Active,' manufactured by Metagenics, as intervention. Each capsule contains 145 mg of the specialized pro-resolving mediators (SPMs) 18-HEPE (25 mg), 17-HDHA (40 mg) and 14-HDHA (80 mg) from fractionated marine lipid concentrate. SPMs are synthesized enzymatically from n-3 polyunsaturated fatty acids (PUFA) known as eicosapentaenoic (EPA) and

docosahexaenoic acids (DHA) and are known to play a critical role in resolving chronic, low-grade inflammation.

1.3 Non-Clinical and Clinical Study Findings

The same 'SPM Active' dietary supplement to be administered to obese subjects in this clinical trial was previously used as intervention in a human clinical trial by Dalli et al, and found to be safe at doses up to 4.5g in healthy individuals.[4] The investigators concluded that SPM supplementation leads to an increase in peripheral blood SPM concentrations and reprograms peripheral blood cells, thus indicating a role for SPMs in mediating immunological activity.[4]

1.4 Relevant Literature and Data

Obesity is a growing national and global epidemic. In the United States; over 35% of Americans are currently obese (Body mass index (BMI) $\geq$30 kg/m^2), and another one-third overweight (BMI: 25-29.9 kg/m^2).[5] Obesity is associated with numerous chronic health conditions, including insulin resistance, type-2 diabetes, hyperlipidemia and hypertension, leading to what is termed as the metabolic syndrome.[6] Chronic inflammation, a central component of obesity, significantly contributes to the development of these metabolic complications and has been associated with accumulation of visceral adipose tissue and immune cell infiltration.[7-9] Recently, the metabolism of immune cells has emerged as a major therapeutic target for improving chronic inflammation and whole-body metabolism, as immune cells have fundamentally different metabolic programming between pro- and anti-inflammatory subsets.[10-13]

Specialized pro-resolving mediators (SPMs) have recently emerged as a target for improving chronic inflammation. SPMs known as protectins, resolvins, lipoxins and maresins, are potent metabolites enzymatically synthesized from n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic (EPA) and docosahexaenoic acids (DHA).[14] Our research, in agreement with other labs, have discovered that metabolites of the SPM family are decreased across a range of tissues in mouse models of obesity and in circulation of obese individuals.[15-18] To exemplify, we have reported that the DHA-derived SPMs 14-HDHA and 17-HDHA are lowered in the spleens of obese C57BL/6J mice.[15] We are also finding a strong reduction in the EPA-derived SPM known as 18-HEPE in white adipose tissue and liver of obese mice relative to lean controls (Fig. 1). Overall, our results are highly consistent with data from a recent study to show that 14HDHA, 17-HDHA, and 18-HEPE are lowered in circulation of obese subjects.[17]

SPMs have insulin/glucose-normalizing properties. Rigorous literature reports demonstrate that EPA- and DHA-derived SPMs improve varying aspects of murine metabolism including insulin resistance and hepatic steatosis.[20-22] We have investigated the effects of resolvin E1 (the downstream product of 18-HEPE) on hyperglycemia and hyperinsulinemia of obese male C57BL/6J mice. Administration of resolvin E1 to obese male mice at 300ng per mouse per day for 4 days prior to sacrifice has no effect on body weight (Fig. 2A) or food intake (data not shown). Resolvin E1 completely restores high fat diet-induced hyperglycemia (Fig. 2B) and strongly improves hyperinsulinemia (Fig. 2C).

2 STUDY OBJECTIVE

The purpose of the study is to determine the efficacy of SPM supplementation on chronic inflammation in obese human subjects. The premise is based on the notion that SPMs are lowered in obese individual. Therefore, it is hypothesized that exogenous administration of SPMs to obese individuals may have therapeutic value as insulin/glucose sensitizing agents by targeting inflammatory pathways.[19] This application breaks new ground by taking the first steps toward translating SPMs from the pre-clinical level to obese

humans. We propose a bioavailability study in which we will establish the concentration of SPMs in plasma and PBMCs of obese subjects (primary aim) in response to dietary supplementation with SPMs. Additional exploratory measurements that correlate metabolic parameters with SPM concentration are proposed in our secondary aim.

2.1 Primary Objective

Our primary objective is to compare immunological fitness pre- and post- oral SPM administration in the obese:

- **Aim 1a**: To quantify SPM concentrations in plasma, serum and PBMCs before and after oral SPM supplementation. We will measure SPMs in 100µl of plasma and 2 x 10^6 PBMCs. PBMCs will be isolated with established protocols in our lab.[15, 26] The rationale for PBMCs vs. plasma is to compare whether SPM levels in PBMCs are higher than plasma or vice versa, indicating SPM uptake into the immune cells or lack thereof. We will also measure other lipid metabolites involved in the inflammatory resolution process including thromboxanes, leukotrienes, prostaglandins, epoxy fatty acids, eoxins, mono-, di-, and tri-hydroxy fatty acids, and hepoxilins. Blinded samples will be shipped to our collaborator Dr. Reisdorph for two-dimensional reverse phase HPLC tandem mass spectrometry.

- **Aim 1b**: To measure antibody responses of B cells in PBMC pool with *in vitro* stimulation and cytokine production before and after SPM supplementation. B cells will be directly stimulated in the PBMC isolate to mimic their natural environment. Then, antibody concentrations will be measured from the supernatants of the cultured cells via ELISA for pre- and post-intervention samples.

2.2 Secondary Objective

Our secondary objective is to compare metabolic profiles in response to an oral dose of SPMs in the obese:

- **Aim 2a**: To measure fasting insulin and fasting glucose before and after intervention. Blood samples from baseline and post-intervention will be used to measure fasting glucose (mg/dL) and fasting insulin (ng/mL) through McClendon labs. Exploratory studies will test if obese adult participants taking a daily dose of SPMs have lower mean fasting levels of serum insulin and glucose after 4 weeks of SPM administration, relative to baseline. Participants will be asked by the study coordinator about the extent to which they complied with dosing at the end of week 2 and week 3 following the start of supplementation

- **Aim 2b**: To correlate pre- and post- metabolic measurements with measured pre- and post- SPM concentrations. Anthropomorphic and blood pressure measurements will be taken. Each subject will have his/her insulin (in ng/dL) and glucose (in mg/dL) measured pre/post supplementation through Family Medicine and the Reisdorph lab will determine SPM concentrations in the plasma pre/post supplementation. Insulin and glucose measurements will then be correlated with measured SPM levels.
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3 INVESTIGATIONAL PLAN (brief overview)

3.1 Study Design

We will conduct a non-randomized uncontrolled clinical trial to measure the efficacy of SPM administration in the obese. We will selectively recruit (n=24) obese subjects (BMI 30-40 kg/m^2) and administer an SPM formulation ("SPM Active") provided by Metagenics for 4 weeks. The study design will rely on blood samples collected at baseline and at the end of the 4-week intervention. All analyses will rely on

comparisons between pre- and post-intervention. The concentration of SPMs, in addition to other PUFA-derived metabolites that share the same enzymatic pathways as SPMs, will be established at baseline and post-intervention using mass spectrometry-based metabololipidomics. The concentration of all metabolites will be measured for plasma and peripheral blood mononuclear cells (PBMCs).

Overview of Study Phases

Screening/Baseline: Subjects will be recruited in person at UNC Family Medicine Center at 540 Manning Drive, Chapel Hill, NC 27599 in a private, closed-door room during a regularly scheduled office visit with Dr. Butler. We are using a convenience sample method, therefore everyone age 50-65 will be screened according to the criteria set forth for the study. During their visit, Dr. Butler will ask subjects if they wish to learn more about the study. If yes, the subjects will be directed to meet with the study coordinator. If the study coordinator is unavailable, Dr. Butler can complete the consent process during the office visit. Recruitment and informed consent will then follow the support documents provided, with either preoperative or post-operative consent and scripts being read by the study coordinator to the potential study subject. Upon full understanding and answering of any questions, the study coordinator will obtain written and verbal informed consent along with providing a copy of the consent form for the subject to keep themselves. We will recruit a total of 24 (n=12 men + 12 women) obese (BMI 30-40 kg/m^2) euglycemic and pre-diabetic subjects who will have similar distributions in their age (50-65 years), race, and smoking status. There will be no randomization for this study.

Intervention/Treatment: All 24 subjects will be administered 4 capsules of 'SPM Active' per day for 4 weeks +/- 2-4 days. There are 3 metabolites of the SPM family in the capsule, which are 17-HDHA (40mg) + 14-HDHA (80mg) + 18-HEPE (25mg). All three metabolites feed into pathways shown to improve insulin resistance and all three metabolites are decreased in obese subjects.[15, 17, 19, 24] The rationale for the dosing and time of intervention is based on the recommendations by the manufacture. The supplement will be provided by Metagenics. Note the manufacturer has not conducted bioavailability studies focused on the obese. Subjects will be instructed to consume 2 capsules with breakfast and 2 with dinner (4 total per day). Fasting blood will be drawn pre- and post-intervention using phlebotomy available under the direction of Dr. Butler.

Baseline measurements will be taken prior to intervention so that each subject may serve as his/her own control. 12-hour fasting blood and serum will be drawn pre- and post-intervention for each subject by a licensed phlebotomist at the UNC Family Medicine Lab and under the direction of Dr. Butler, the co-I. Each blood sample will be the size of 10 10mL plasma and serum tubes (9 plasma tubes + 1 serum tube) to ensure that enough B cells are collected from PBMCs for activations (see section 4.1 below). Therefore, a total of 10 tubes or ~100 ml of blood will be drawn twice during the study, 4 weeks apart. Finally, each subject will have his/her height (ft), body weight (lbs), blood pressure (mmHg), and pulse (bpm) taken at baseline and 4 weeks of intervention.

Follow up: Subjects will have two face-to-face visits and two phone contacts during the study. The first visit will be at baseline (pre-intervention) and the second visit will conclude the study (post-intervention). We will continuously monitor subject health status throughout the study via phone contact at the end of week 1 and week 3 following the start of supplementation. At the end of the study, all patients will follow up in a routine manner as designated by their physician at UNC Family Medicine.

Unscheduled Visits: If the study coordinator relays health concerns to Dr. Butler that will necessitate in-person visit.

3.2 Allocation to Treatment Groups and Blinding (if applicable)

There will be no randomization or blinding for this study. All 24 subjects will receive the "SPM Active" intervention. Baseline measurements will be taken prior to intervention so that each subject may serve as his/her own control.

3.3 Study Duration, Enrollment and Number of Subjects

Each subject's participation will last up to 4 weeks +/- 2-4 days, with no additional time involved in this study outside normal pre- and post-operative care. The entire study is expected to last 6-9 months. This includes an enrollment period of 3-4 months and data analysis.

3.4 Target Population

Inclusion Criteria:

- Male and female subjects with age 50-65 years and obese (i.e. BMI 30-40) who are euglycemic and pre-diabetic (i.e. fasting glucose 70-125 mg/DL or HbA1c of 5.7-6.4%).
- Only post-menopausal females will be recruited in the female cohort to reduce the confounding inflammatory effects of estrogen with supplementation.

Exclusion Criteria:

- Those with fasting glucose values > 126 mg/dL or known type 2 diabetes
- Females who are pre-menopausal, pregnant, planning to become pregnant, breastfeeding or lactating
- Subjects consuming n-3 PUFA supplements in the last 3 months prior to enrollment, high consumption of fatty fish (>2 servings per week), and subjects with active autoimmune disease, liver disease, coagulopathy, hypothyroidism, known allergy to fish or shellfish, inability to give informed consent, or taking anticoagulants (e.g. warfarin and direct acting anticoagulants), those taking estrogen or testosterone, and anyone taking daily aspirin, NSAIDs, or active asthma medications.
- Subjects receiving immunomodulatory or immunosuppressant therapy (corticosteroids or monoclonal antibodies) in the 4 weeks prior to study enrollment, and subjects with known active malignancy or undergoing treatment for malignancy will be excluded.

Demographics: No exclusions will be made based on race, gender, or ethnicity. Since all female subjects will be post-menopausal, this study will not include female subjects who are pregnant or become pregnant. This is not because we expect risk to mother or fetus in pregnancy but rather because of the hormonal effects of high estrogen in pre-menopausal women on the lipidomic profile that we are studying. Moreover, the inflammatory effects of estrogen will confound the lipidomic profile measured from the subjects. For this reason, the female cohort will include only post-menopausal women.

4 STUDY PROCEDURES

4.1 Screening/Baseline Visit procedures

Insulin (ng/dL), glucose (mg/dL), BMI (kg/m^2), weight (lbs), height (ft), disease status, and medications will be abstracted from medical charts. These metabolic measurements will be compared pre- and post-

supplementation for aim2a, and used to investigate their association with SPM concentrations for aim2b.

12-hour fasting blood and serum will be drawn for each subject by a licensed phlebotomist at the UNC Family Medicine Lab and under the direction of Dr. Butler at baseline. Each blood sample will be the size of 10 10mL plasma and serum tubes (9 plasma tubes + 1 serum tube). PBMCs will be isolated from each blood sample. SPM concentration will be compared in the plasma, serum and PBMCs obtained for each patient, in accordance with aim 1a. For aim 1b, we will plate 15 wells of B cells (isolated from the PBMCs) for activations with 200,000 B cells per well (3 x 10^6 total). We are assuming 1 mL blood yields approximately 1 x 10^6 PBMCs. We also assume 0.5 x 10^6 PBMCs per well with 5% B cells. Since, on average, 7-8 mL of blood is collected per 10mL tube, we will collect 10 tubes to ensure we have at least 60 mL blood for the 60 x 10^6 PBMCs needed to collect 3 x 10^6 B cells for activations. Therefore, a total of 10 tubes or ~100 ml of blood will be drawn.

In addition to blood collection, each subject will have his/her height (ft), body weight (lbs), blood pressure (mmHg), and pulse taken (bpm).

4.2 Intervention/Treatment procedures

All subjects will receive the dietary supplement "SPM Active", provided free of cost by Metagenics. Subjects will be responsible for taking 4 capsules per day (2 at breakfast and 2 at dinner) for a total of 4 weeks.

4.3 Follow- up procedures

There is no scheduled follow up procedure apart from that detailed in the study design. The patients will follow up in a routine manner as designated by their physician at UNC Family Medicine.

4.4 Unscheduled visits

The study coordinator may relay health concerns to Dr. Butler that will necessitate an in-person visit.

4.5 Concomitant Medication documentation

All prescription or OTC drugs taken by study subjects will be examined by Dr. Butler and his team prior to enrollment in the study. No previous adverse events have been observed in previous studies (such as the human clinical trial done by Dalli et al.) with "SPM Active," though we will continuously monitor subject health status via phone contact at the end of week 1 and week 3.

4.6 Subject Completion/ Withdrawal procedures

Subjects will be compensated $250 cash for their participation and completion of the study ($100 at baseline visit + $150 at week 4 visit upon completion). No prorated compensation will be applied for this pilot study. This amount of compensation is not deemed coercive; the incentive is meant to compensate subjects for their time in the study which include two study visits, two phone visits, and two blood draws.

This is a voluntary research study. Refusal to participate or withdrawal of participation and/or consent can occur at any time, for any reason. Subjects may be withdrawn if: (1) they experience intolerable adverse reactions or allergic reaction to study supplement or (2) are not at least 90% compliant with taking study supplement during the 4 weeks active phase. In the event of withdrawal, all personal health information (PHI), electronic or hard copy, will be immediately purged and permanently destroyed. Withdrawal or

refusal will not influence ability, timing, or quality of clinical care, type of clinical care administered, standing with UNCHC system, or ability to participate in future research studies. The investigators also have the right to stop participation at any time. This could be because participants have chosen to stop supplementation, or have failed to follow instructions or because the entire study has been stopped.

4.7 Screen failure procedures

Study participants will be checked to determine if they meet the inclusion and exclusion criteria. Those that are not eligible will be excluded from the study. If the study coordinators are not able to contact study subjects in the follow-up phone calls they will be considered as "loss to follow-up" and no longer enrolled in the study.

5 STUDY EVALUATIONS AND MEASUREMENTS

Samples taken from pre- and post-intervention visits will be immediately transferred to Michael Hooker Research Center Room 2204 (Shaikh Lab) for immediate processing. Tubes will be labelled with a de-identified research code and will not include any PHI. We will measure SPMs in 100µl of plasma and serum and $2x10^6$ PBMCs. PBMCs will be isolated with established protocols in our lab [1, 2.] The rationale for PBMCs vs. plasma and serum is to compare biomarkers for SPM uptake. We will also measure other lipid metabolites including thromboxanes, leukotrienes, prostaglandins, epoxy fatty acids, eoxins, mono-, di-, and tri-hydroxy fatty acids, and hepoxilins. Blinded samples will be shipped to our collaborator Dr. Reisdorph (pay-for-service, see support letter) for two-dimensional reverse phase HPLC tandem mass spectrometry. Blood samples from baseline and post-intervention will be used to measure fasting glucose (mg/dL), fasting lipid panel (total cholesterol in mg/dL, LDL in mg/dL, HDL in mg/dL, triglyceride levels in mg/dL) and fasting insulin (ng/mL) through McClendon labs.

5.1 Efficacy Evaluation

There will be no efficacy evaluation due to the pre-/post- study design, which requires that data be obtained through the end of the clinical trial. However, the study may be stopped due to safety concerns (see safety evaluation section 5.2).

5.2 Safety Evaluations

Any health conditions that arise over the course of the study that were not present in the subjects prior to their enrollment will be monitored by Dr. Butler who will evaluate whether to withdraw the subject from the study.

6 STATISTICAL CONSIDERATION

6.1 Primary Endpoint

The primary aim of the study is designed and powered to measure an increase in 18-HEPE, 17-HDHA, and 14-HDHA in Aim 1. At a family-wise error rate of 5% controlling for testing of three metabolites mentioned above, n=9 will provide more than 80% power to detect a 50%-fold change increase in metabolite levels within each subject. We are assuming a similar effect size for both males and females and are doubling the sample size (n=18, 9 per sex) to allow us to subset the data in case we find divergent results between sexes. To account for possible loss-to-follow up, we increased the sample size from 18 to 24 (n=12 male + 12 female). Power analysis was conducted assuming a paired t-test on pre- and post-treatment metabolite levels for three pre-specified metabolites. Expected variability of within subject differences in metabolite levels was assessed from a previous dataset and the median standard deviation was calculated across all

metabolites.[1] Power analyses were calculated by Dr. Michael Love in the UNC Department of Biostatistics. Note that all analyses will be conducted in the R statistical software.

- Aim 1a: SPM lipidomic measurements will be analyzed pre/post supplementation. Each SPM metabolite's distribution will be assessed for normality and homogeneity of variance using the Shapiro-Wilks test and Bartlett test respectively. However, normality will also be assessed via a qqplot of the standardized residuals and we will visualize the distribution of our data with a histogram. In addition, to corroborate the Bartlett test we will check the homogeneity of variance of the residuals (homoscedasticity) using a scale-location plot where the square roots of the absolute values of the standardized residuals are plotted. If the metabolite's distribution satisfies the assumption of normality & homogeneity of variance then a paired two-tailed Student's T-test will be used to generate p-values corresponding to differences before and after supplementation. If the assumption of heteroscedasticity is not met, the data will be analyzed using a paired Welch T-test. Another alternative would be to apply a log or square root transformation. However, if the data still fails the assumptions of normality and heteroscedasticity it will be analyzed with a paired Wilcoxon rank sum test. All p-values will undergo a Benjimani-Hocherberg adjustment (optimal for large-scale multiple hypothesis testing) at a false discovery rate of 5% to account for multiple hypothesis testing between metabolites.

- Aim 1b: Antibody concentrations measured via ELISA will be tested for the assumptions of normality and homogeneity of variance using the Shapiro-Wilks test and Bartlett test respectively. However, normality will also be assessed via a qqplot of the standardized residuals and we will visualize the distribution of our data with a histogram. In addition, to corroborate the Bartlett test we will check the homogeneity of variance of the residuals (homoscedasticity) using a scale-location plot where the square root of the standardized residuals is plotted. Data that passes both assumptions will be analyzed via a paired two sample Student T-test. Data that fails the assumption of heteroscedasticity will be analyzed using a paired Welch T-test. Another alternative would be to apply a log or square root transformation. However, if the data still fails the assumptions of normality and heteroscedasticity it will be analyzed with a paired Wilcoxon rank sum test. All p-values from these analyses will undergo a Bonferroni p-value adjustment (optimal for smaller-scale multiple hypothesis testing).

6.2 Secondary Endpoint

- Aim 2a: The data will be assessed for normality and homogeneity of variance using the Shapiro-Wilks test and Bartlett test respectively. However, normality will also be assessed via a qqplot of the standardized residuals and we will visualize the distribution of our data with a histogram. In addition, to corroborate the Bartlett test we will check the homogeneity of variance of the residuals (homoscedasticity) using a scale-location plot where the square root of the standardized residuals is plotted. Data that passes both assumptions will be analyzed via a paired two sample Student T-test. Data that fails the assumption of heteroscedasticity will be analyzed using a paired Welch T-test. Another alternative would be to apply a log or square root transformation. However, if the data still fails the assumptions of normality and heteroscedasticity it will be analyzed with a paired Wilcoxon rank sum test. All p-values from these analyses will undergo a Bonferroni p-value adjustment.

- Aim 2b: The data will also be assessed for normality using the Shapiro-Wilks test, qqplots, and we will visualize the distribution of the data with a histogram. If the data are normal or it cannot be transformed to approximate a Guassian distribution, we will utilize a Pearson correlation to

determine associations between SPM concentration and metabolic outcomes (insulin/glucose measures). If the data is not normal we will utilize a Spearman correlation. All p-values from these analyses will undergo a Bonferroni p-value adjustment.

6.3 Statistical Methods

Data will be assessed for normality and homogeneity of variance using the Shapiro-Wilks test and Bartlett test respectively. However, normality will also be assessed via a qqplot of the standardized residuals and we will visualize the distribution of our data with a histogram. In addition, to corroborate the Bartlett test we will check the homogeneity of variance of the residuals (homoscedasticity) using a scale-location plot where the square root of the standardized residuals is plotted. Data that passes both assumptions will be analyzed via a paired two sample Student T-test. Data that fails the assumption of heteroscedasticity will be analyzed using a paired Welch T-test. Another alternative would be to apply a log or square root transformation. However, if the data still fails the assumptions of normality and heteroscedasticity it will be analyzed with a paired Wilcoxon rank sum test. All p-values will undergo either a Bonferroni or Benjimani-Hocherberg p-value adjustment.

Further analyses include determining patterns of metabololipidomic signatures and immune cell phenotypes from both aims. To do this we will conduct a principal component analysis (PCA) on the metabololipidomics and ex-vivo B cell antibody production data. PCA will aid in reducing the biological noise in the dataset and identify trends between metabolites and B cell function to determine correlated metabololipidomic signatures. To identify clusters of signatures shared among individuals, k-means clustering on the PCA analysis will be conducted to visualize the partitioned metabolite-cellular landscapes pre- and post-intervention. Moreover, statistical estimates of population parameters will be tabulated along with corresponding confidence intervals (CIs) and/or standard errors (SEs) to convey levels of precision / imprecision.

6.4 Sample Size and Power

Experimental rigor is ensured through the analyses of blinded samples. The study is designed and powered to measure an increase in 18-HEPE, 17-HDHA, and 14-HDHA in Aim 1. Additional measures in Aim 2 are exploratory, but necessary for future grant applications to demonstrate the feasibility of the approach. At a family-wise error rate of 5% controlling for testing of three metabolites mentioned above, n=9 will provide more than 80% power to detect a 50%-fold change increase in metabolite levels within each subject. We are assuming a similar effect size for both males and females and are doubling the sample size (n=18, 9 per sex) to allow us to subset the data in case we find divergent results between sexes. To account for possible loss to follow-up, we will increase sample size from 18 to 24 (n=12 male + 12 female). Power analysis was conducted assuming a paired t-test on pre- and post-treatment metabolite levels for three pre-specified metabolites. Expected variability of within subject differences in metabolite levels was assessed from a previous dataset and the median standard deviation was calculated across all metabolites.[1] Power analyses were calculated by Dr. Michael Love in the UNC Department of Biostatistics.

6.5 Interim Analysis

Given the pre-/post- design of the study, doing an interim analysis in the middle of the study is not feasible, for we will have to obtain and compare pre- and post- data. Additionally, the metabolipidomics analyses that

we are performing will take several months to complete. Finally, with the small number of subjects, this study is not expected to span a long period of time.

7 STUDY INTERVENTION

The "SPM Active" supplement will be shipped all at once from Metagenics at room temperature to the UNC Family Medicine clinic with expiration that covers the full duration of the intervention. Study participants will be instructed to keep the supplement at room temperature. Subjects who have consented to the study will receive the exact number of tables required for the study. They will be instructed to consume 4 capsules per day (2 capsules with breakfast and 2 with dinner) of "SPM Active" for 4 weeks. Each capsule contains 145 mg of SPMs for a total daily dose of 580 mg. There are 3 metabolites of the SPM family in the capsule, which are 17-HDHA (40mg) + 14-HDHA (80mg) + 18-HEPE (25mg). All three metabolites feed into pathways shown to improve insulin resistance and all three metabolites are decreased in obese subjects.[15, 17, 19, 24] The rationale for the dosing and time of intervention is based on the recommendations by the manufacturer. Appropriate dosing will be monitored with the follow-up phone calls at weeks 2 and 3. Participants who have chosen to stop supplementation, or have failed to follow supplement instructions will be withdrawn from the trial.

8 STUDY INTERVENTION ADMINISTRATION

No randomization or blinding/unblinding procedures will be used for study.

9 SAFETY MANAGEMENT

Safety standards will be overseen by Co-Investigator, Dr. Erik Butler, DO and his designated study coordinator. Subjects will be screened for adverse events during two follow-up phone calls at Weeks 1 and 3 of intervention, as well during last study visit, Week 4. Subjects will be given the study coordinator's phone number to report any Adverse Events or Serious Adverse Events. All reported Adverse Events (AEs) will be given to the co-investigator, Dr. Butler, each week of the study.

Should a Serious Adverse Event (SAE) occur, Dr. Butler, co-investigator, will be immediately notified. The subject will be instructed to stop study drug, and seek immediate medical attention. SAEs include but are not limited to bleeding, chest pain/pressure, shortness of breath, weakness, altered mental status, vision changes or extreme pain.

A log of all AEs will be maintained throughout the study and will be assigned to each patient. Note that this study will not require a Data Safety Monitoring Board (DSMB) due to its small sample size and the low risk of study drug. However, Dr. Erik Butler, co-Investigator, will review weekly the adverse event reporting log for all subjects. Any clinically significant AEs will be follow-up by Dr. Butler and the study coordinator to ensure resolution. Patients will be further evaluated by Dr. Butler or other health care professional for ongoing AEs.

Though highly unlikely, if 50% or more of total study participants report intolerable adverse reactions from taking the supplement, we will stop the study.

10 DATA COLLECTION AND MANAGMENT

Personal identifiers and medical history will be collected following patient recruitment and informed consent.

Data from this study will be kept in electronic and hard copy forms with the respective security measures. Electronic data will be kept on an encrypted desktop computer protected by multiple passwords in a lab, located on a hall protected by card access in Michael Hooker Research Center, Chapel Hill, NC. De-identified data will be stored on REDCap for use by research team personnel only. Data management will be performed by the PI and the study coordinators. All data acquisition will meet HIPPA and confidentiality standards. Electronic data will be stored for a minimum of five years in the case that this data needs to be verified. When deemed appropriate, all files will be purged and permanently deleted from research computers. Hard copy data will be shredded. Human biological samples (serum/plasma) will be stored for a minimum of five years in the case that these samples are needed for duplication of or verification of study results. Please see Part C for further details. These samples will be stored in locked freezers in locked research laboratories in secure facilities that require card access. When all analysis of human biological samples is completed, all samples will be disposed of according to university and environmental health guidelines. Data that links dummy identifiers to any personal identifying information will be shredded and disposed of, or, in the case of electronic data, will be permanently deleted. Limited clinical data (glucose, insulin, lipid panel) may be part of subjects' secure electronic medical record in Epic.

Note that the biological samples and data collected from subjects who are lost to follow-up will be handled in this way as well.

The PIs will be responsible for monitoring and reviewing subject data, lab results, study progress, subject safety and confidentiality, and the accuracy and security of the emerging data. They will also be responsible for running the assays and entering all data into redcap for statistical analysis.

11 RECRUITMENT STRATEGY

Subjects will be recruited at UNC Family Medicine Center at 540 Manning Drive, Chapel Hill, NC 27599. Dr. Butler and the study coordinator will be responsible for recruiting subjects. We are using a convenience sample method, therefore everyone age 50-65 years will be screened according to the criteria set forth for the study. Subjects will be asked questions orally during the primary visit to determine if eligibility criteria are met, at which time informed consent and HIPPA authorization will be obtained. Recruitment will take place in a private room during a normally scheduled clinical visit. Subjects will be briefed on the scientific premise of the study along with details on participation involvement, risks, benefits to the individual, benefits to scientific knowledge, ability to refuse participation and/or ability to withdraw participation at any time.

12 CONSENT PROCESS

Subjects will be asked questions orally during the primary visit to determine if eligibility criteria are met, at which time informed consent and HIPPA authorization will be obtained. Subjects will be recruited and consented in a private, closed-door room with preoperative or post-operative consent and scripts being read by the study coordinator to the potential study subject. Upon full understanding and answering of any questions, the study coordinator will obtain written and verbal informed consent along with providing a copy of the consent form for the subject to keep themselves. Dr. Butler or the study coordinator will consent subjects in a professional and non-coercive manner.

13 PLANS FOR PUBLICATION

If our hypothesis is correct, data from aims 1 and 2 will provide a solid foundation for an NIH R21 grant. The lead PI, after consultation with the others on the investigative team, will communicate with program officials to tailor an R21 grant to NIDDK PA-18-104 that would focus on the mechanistic links between adipose tissue pro-resolving lipid mediators and lymphocyte metabolism.

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15 APPENDIX

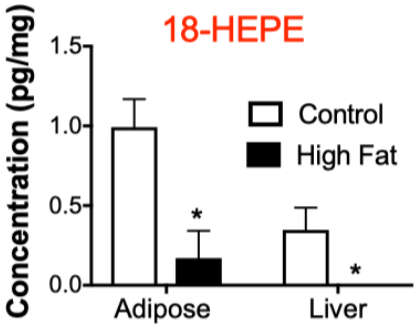

Fig. 1: A metabolite of the EPA-derived SPM family is lowered in obese mice. C57BL/6J male mice were administered a lean control or high fat diet for 15 weeks. Visceral white adipose tissue and liver were subjected to mass spectrometry based metabololipidomics for PUFA-derived mediators. For simplicity, we present 18-HEPE. Data are average ± SEM from 5 mice per diet. *$p<0.05$. Experiments with females are underway. Analyses were conducted blinded to ensure strong rigor.

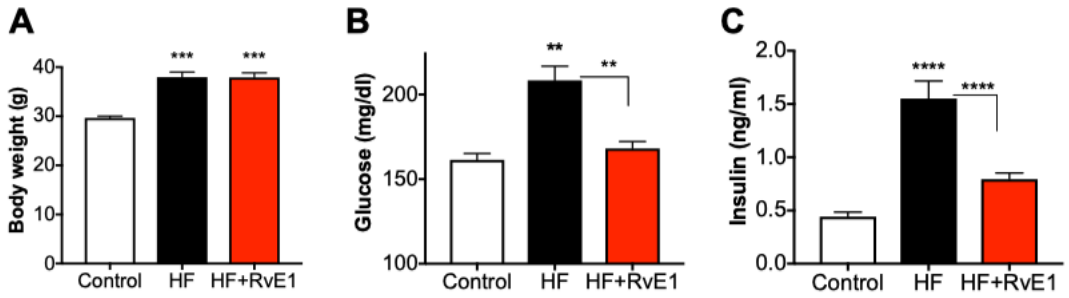

Fig. 2: Short-term administration of EPA-derived SPM improves fasting glucose/insulin levels of obese mice. Male C57BL/6J mice were fed as described in Fig.1. Prior to sacrifice, a cohort of mice was i.p. injected daily for 4 days with 300ng resolvin E1 or vehicle control. (A) Body weights, (B) fasting glucose, and (C) fasting insulin levels. Data are average + SEM from 11-18 mice per diet. **p<0.01, ***p<0.001, ****p<0.0001.