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TITLE: Developing Treatment, Treatment Validation & Treatment Scope in the Setting of an Autism Clinical Trial

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14. ABSTRACT We obtained IRB approval on December 7th., 2009.. We applied for and received a COC from the NIH as required and received final approval from our IRB on March 25th 2010. The test material DHA and the placebo were acquired from Martek with a significant and unforeseen delay August 20th 2010 (please see Partnering project W81XWH-08-1-0730, Task #2). The Data Safety Monitoring Board and all other requirements for subject recruitment have been completed. Additional sources of subjects are being arranged to make up for delays occurring in obtaining IRB approval and issues obtaining the material from Martek (please see Partnering project W81XWH-08-1-0730, Tasks #1 and #2). We have established a set of LC-MSMS conditions appropriate for looking the presence of LXA4, RvD1, RvE1 and Maresin in urine. The necessary standards in natural and deuterated form have been prepared by total organic synthesis. We have established a set of LC-MSMS conditions appropriate for looking the presence of LXA4, RvD1, RvE1 and Maresin in urine. . Due to our late start we applied for and received a no-cost extension for this project. The SOW was updated on March 22nd 2010. and has been updated for the no-cost extension. Tasks remain the same only the timeframes in which they will be completed has changed. Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730					
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Introduction:

This project is to test to see if DHA treatment can beneficially affect excretion of urinary biomarkers of oxidative stress and the autism clinical phenotype. In addition polymorphic variants of genes of certain enzymes that synthesize and metabolize docosahexaenoic acid (DHA) may contribute to the phenotype of some autism cases. We will test to see if any of these genes are risk factors for autism. We will also measure changes in excretion of the polyunsaturated fatty acid (PUFA) derived biomarkers of oxidative stress (isoprostanes and neuroprostanes) together with the changes in production of anti-inflammatory lipid mediators. We will test these biomarkers to see if we can monitor and validate effectiveness of DHA therapy. We will also test the genotypes of key DHA-metabolizing enzymes can predict which patients will respond to therapy Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Body:**PROJECT #2: PI T.P. STEIN, PhD, PARTNERING PI, W81XWH-08-1-0729**

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Unless otherwise stated, tasks are divided between the synthetic core (directed by Dr. B.W. Spur) and the Analytical core (directed by Dr. T.P. Stein).

Task #1 Obtain IRB approval (Drs. Stein and Spur).

Our latest Continuing Review was approved on September 29th 2011. In addition a restructuring of partnering project W81XWH-08-1-0730 was done along with related changes to the protocol to help increase the rate of subject recruitment. These changes were also approved by our IRB on September 29th 2011. The approved CR was sent to the HRPO on October 1st 2011. Please see partnering project W81XWH-08-1-0730 Tasks #1 and 2 for detailed description.

Task #2 After IRB approval has been obtained and permission given to proceed we (B.W. Spur and A. Rodriguez) will chemically synthesize the protective lipid metabolites: (i) Lipoxin A4 (LXA4, 5S, 6R,15S-trihydroxyl-7, 9,13-trans-11-cis-eicosatetraenoic acid), (ii) lipoxin A4 precursor, 15-hydroxy-eicosatetraenoic acid, (iii) Resolvin D1 (RvD1, 7S,8R,17S trihydroxy-4Z,9E,11E,13Z,15E, 19Z-docosahexaenoic acid) (iv) Resolvin D precursor (17-hydroxy-docosahexaenoic acid), (v) Resolvin E1 (RvE1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16 Eicosapentaenoic acid and (vi) Protectin D1 (10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E, 15Z,19Z-hexaenoic acid) all with and without a deuterium label. It

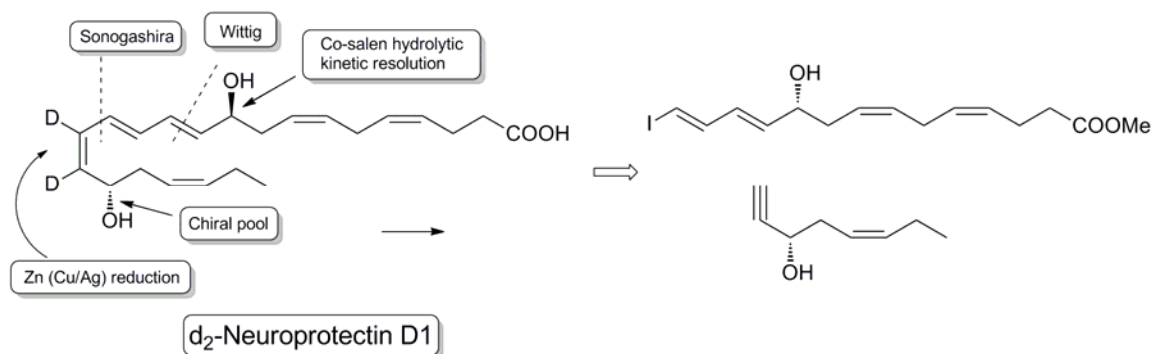
is expected that task #2 will take two years ((21-42 months) as stated in the current SOW).

Highlights of the year: The total syntheses of Neuroprotectin and Maresin in the natural and isotopically labeled form has been achieved. A new general strategy has been developed starting from arabinose that allows the syntheses of all resolvins from either D (-) arabinose for the S-chirality or L (+) arabinose for the R-chirality of hydroxy groups. Both starting materials are readily available on the molar range.

1. COMPLETION OF THE SYNTHESIS OF DI-DEUTERIO-NEUROPROTECTIN

As stated in the previous progress report, Dr. Spur and his colleague Dr. Rodriguez were working on these syntheses. The synthesis of Di-deuterio-Neuroprotectin (d₂-NPD1) has now been completed.

Synthesis of Di-deuterated-Neuroprotectin D1

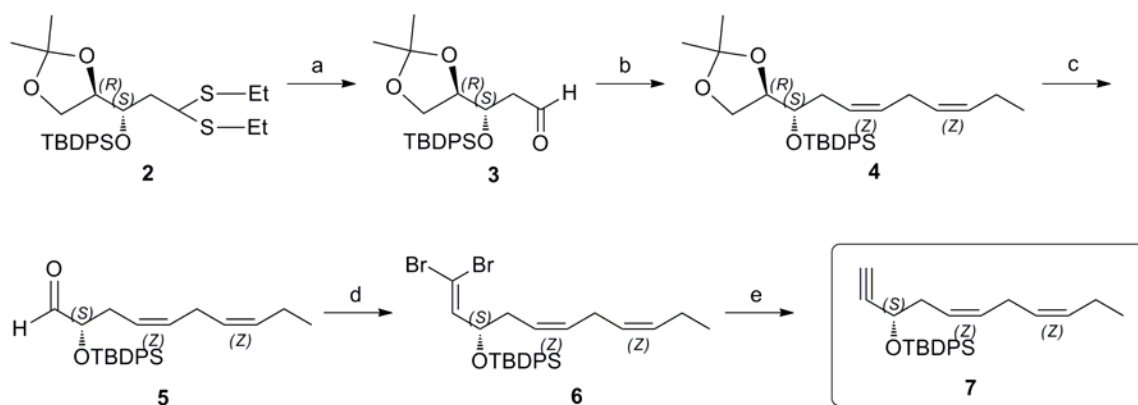


The synthesis of Di-deuterated Neuroprotectin D1 was accomplished by the coupling of the two key fragments via a Pd/Cu catalyst as outlined above. Initial difficulties were observed due to the source of the palladium catalyst. This issue has been resolved using another source of the palladium catalyst. A cis-selective Deuterium reduction of the triple bond by the Zn/Cu/Ag method using D₂O / d₄-methanol provided the target molecule. The stereocenter at carbon 10 was obtained via a hydrolytic kinetic resolution according to the Jacobsen's method with high enantiomeric excess followed by a selective primary silylether oxidation with the Swern reagent. A Wittig coupling with triphenylphosphoranylidene-acetaldehyde followed by the Takai reaction (CHI₃ / CrCl₂) gave the key intermediate. The second chiral center was obtained using the chiral pool strategy starting from D-(-)-arabinose. The simultaneous cleavage of the TES and TBDP groups was cleanly achieved with tetrabutylammonium fluoride in THF.

2. COMPLETION OF THE SYNTHESIS OF MARESIN

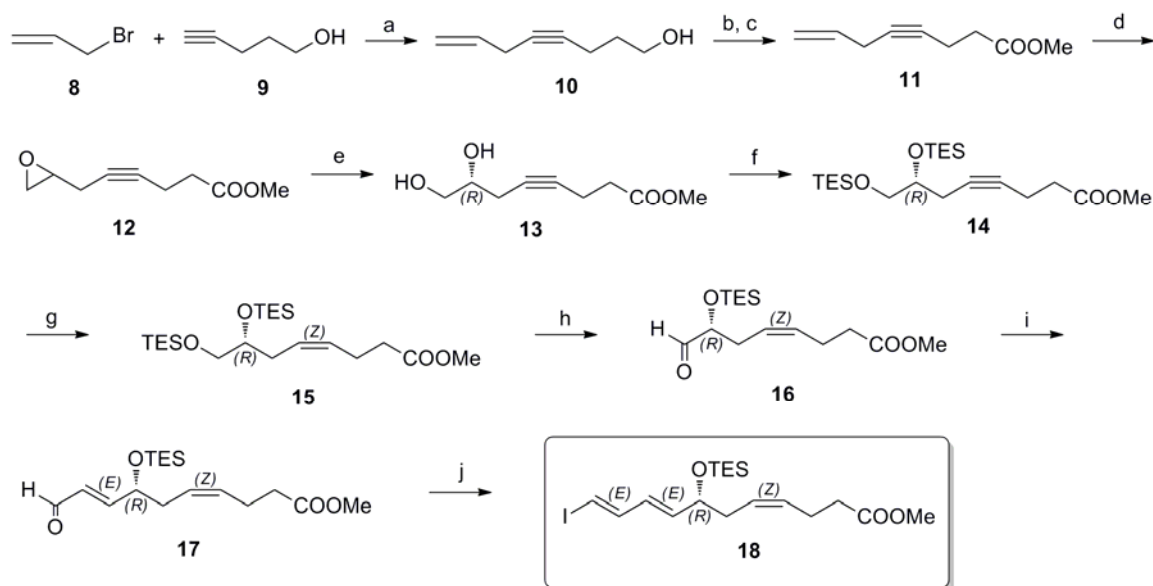
The total synthesis of Maresin is outlined in scheme 1, 2 and 3. The chiral fragment **7** was constructed using the chiral pool strategy starting from the TBDPS protected D-(-) arabinose dithioacetal derivative **2** (scheme 1). The second chiral key intermediate **18** was obtained using the Jacobsen's hydrolytic kinetic resolution (scheme 2). As shown in

scheme 1 deprotection of the dithioacetal groups of **2** with NCS, AgNO₃ liberates the protected aldehyde **3** that was reacted with the cis-3-hexenyl-triphenylphosphorane to give the intermediate **4**. Isopropylidene and diol cleavage of **4** was accomplished in one step following the Rokach protocol with periodic acid in THF/ether at room temperature to give **5** in high yield. The synthesis of the key fragment **7** was completed by the Corey-Fuchs homologation procedure.



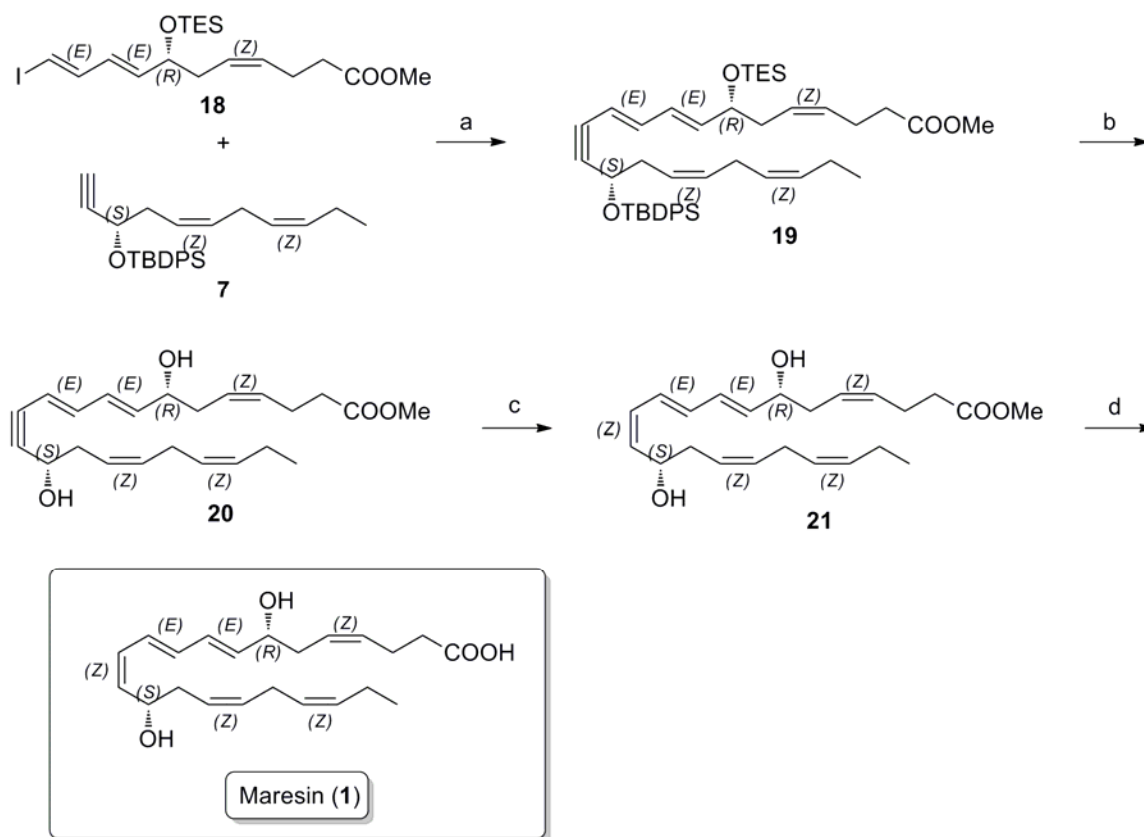
Scheme 1. *Reagents and conditions:* (a) NCS, AgNO₃, CH₃CN then DMSO; (b) CH₃CH₂CH=CH(CH₂)₂P⁺Ph₃I⁻, BuLi, THF, -78°C ; (c) periodic acid, THF:Et₂O;(d) CBr₄, Ph₃P, CH₂Cl₂, 0°C; (e) LDA, THF, -78°C then CH₃COOH.

The second key fragment **18** (scheme 2) was obtained from allyl bromide (**8**) and 4-pentynol (**9**) in water. Jones oxidation of **10** followed by esterification with methanol / dimethoxypropane cat. TMS-Cl at room temperature gave **11** in quantitative yield. Epoxidation with m-chloro-perbenzoic acid gave the racemic epoxy ester **12**. Hydrolytic kinetic resolution with the S,S-Co-salen complex gave the chiral diol ester **13** that was protected with TES-chloride to give **14**. Palladium-Lindlar (Fluka) semihydrogenation produced cleanly the cis-alkene **15**. Selective oxidation of the primary silyl ether with the Swern reagent gave the aldehyde **16**. Wittig homologation with triphenylphosphoranylidene-acetaldehyde followed by Takai reaction (CHI₃ / CrCl₂) gave the key intermediate **18**.



Scheme 2. *Reagents and conditions:* (a) Na_2SO_3 , cat. CuI , K_2CO_3 , H_2O ; (b) Jones reagent, acetone; (c) 10% TMSCl , MeOH , 2,2-dimethoxypropane, rt; (d) MCPBA , NaHCO_3 , CH_2Cl_2 , 0°C ; (e) $(S,S)\text{-(salen)Co(III)(OAc)}$ catalyst, H_2O , 0°C to rt.; (f) TESCl , imidazole, Et_3N , DMF ; (g) H_2 , Lindlar cat., hexane; (h) $(\text{COCl})_2$, DMSO , CH_2Cl_2 then Et_3N ; (i) $\text{Ph}_3\text{P=CH-CHO}$, benzene, 70°C ; (j) CrCl_2 , CHI_3 , THF , 0°C to rt.

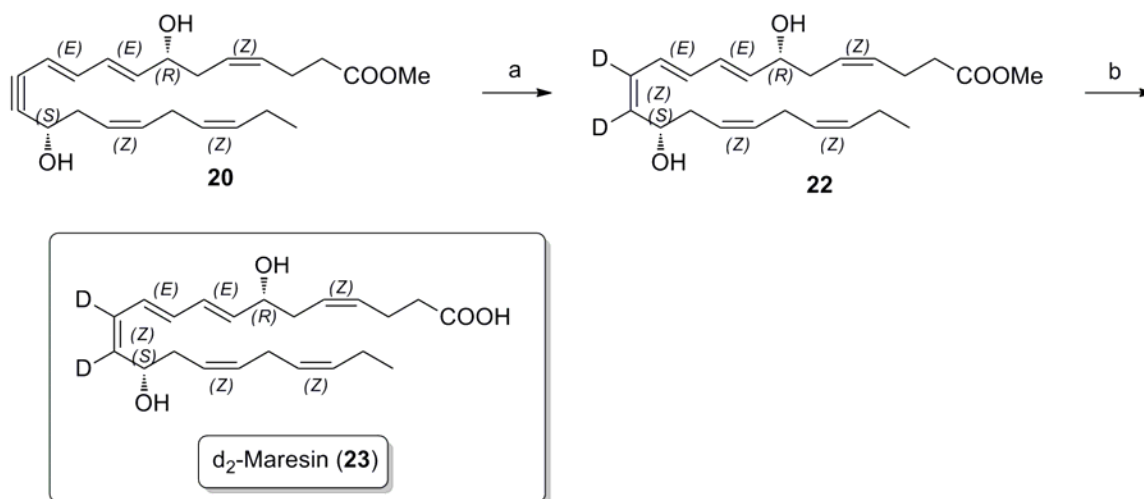
The total synthesis of Maresin was completed as shown in scheme 3. Sonogashira coupling of the two key fragments **7** and **18** with cat. $\text{Pd(PPh}_3)_4$, CuI in benzene at room temperature gave the silyl protected acetylene precursor of Maresin (**19**). Desilylation with tetrabutylammonium fluoride in THF followed by cis-selective reduction of the conjugated triple bond gave Maresin methyl ester (**21**). Mild alkaline hydrolysis with 1N LiOH in methanol/ H_2O followed by acidification with phosphate buffer in the presence of ethyl acetate gave natural Maresin (**1**).



Scheme 3. *Reagents and conditions:* (a) $\text{Pd}(\text{PPh}_3)_4$, CuI , $n\text{-PrNH}_2$, benzene, rt; (b) TBAF, THF, 0°C to rt; (c) $\text{Zn}(\text{Cu/Ag})$, aq. CH_3OH , $40\text{--}50^\circ\text{C}$; (d) 1N LiOH , $\text{MeOH}:\text{H}_2\text{O}$, 0°C , then H^+ pH 5.5.

SYNTHESIS OF DI-DEUTERIO-MARESIN

The acetylene precursor for Maresin **20** served as the starting material for the synthesis of the di-deuterated Maresin (Scheme 4). Using our protocol for the cis reduction in D_2O / CD_4OD in the presence of freshly prepared Zn/Cu/Ag under Argon at 45°C gave cleanly the di-deuterated Maresin methylester **22**. Mild alkaline hydrolysis with 1N LiOH in methanol/ H_2O followed by acidification with phosphate buffer in the presence of ethyl acetate gave natural d2-Maresin (**23**).



Scheme 4. Reagents and conditions: (a) Zn(Cu/Ag), D₂O/CD₃OD, 40–50°C; (b) 1N LiOH, MeOH:H₂O, 0°C, then H⁺ pH 5.5.

Task #3 (i) Year 1 and the first half of year two will be spent in developing isotope dilution LC-MSMS assays for the compounds referenced in task #2 (T.P. Stein and technician). Also to be done during that year are to replicate in our laboratory published LC-MSMS assays for isoprostane and isoprostane metabolites in urine, 2,3 dinor-5,6 dihydro-PGF_{2t} and iPF₄α-VI (T.P. Stein and technician).

The objectives of this years research were:

(1) DETECTION OF COMPOUNDS IN PRE-EXISTING URINE SAMPLES.

(1-a). Results: During the first half of the year we used a pre-existing series of 20 urines we investigated which of the compounds of interest RvD1, RvE1, LXA4, 12-HETE could be reliably determined in urine. The results are shown in the table below. For 12-HETE and LXA4 we found no free compound in the 20 test samples so only the total (after enzyme treatment) data is given in the table. The data shows that we are able to detect and quantify LXA4, Maresin and 12-HETE in urine. RvD1 was seen in only one urine and RvE1 was not found. These studies were done before Dr. Spur provided us Neuroprotectin,

TABLE 1

	MARESIN TOTAL. pg mg CREAT	12-HETE TOTAL ng mg CREAT	LXA4 TOTAL ng mg CREAT	MEHP, TOTAL ng mg CREAT
MEAN	141	750	801	33
SEM	46	368	316	9
N	16	12	9	20

(2). ANALYTICAL CONDITIONS FOR NEUROPROTECTIN

(2-a): Results: During the year Dr Spur completed the syntheses of Neuroprotectin D1 with and without deuterium labeling. We have determined its elution pattern and molecular reaction mechanisms (MRM's). Under the LC conditions we expect to use for the Autism children's samples, NPD1 elutes at 18.39 minutes with MRM transitions at 359.2 > 153.2 and 359.2 > 205.8. We now have operative an analytical program for detecting all of the compounds of interest.

(3) DETERMINE THE STABILITY OF THE COMPOUNDS OF INTEREST IN URINE.

It is important to know how stable the compounds of interest are when stored at -70°C because it is technically impossible to exam each urine immediately after collection. We decided not to wait for completion of the NP syntheses and went ahead with 4 test urines doped with RvD1, RvE1, Maresin, LX4 and 12-HETE. Test samples were stored at -70°C. If there is loss with urine storage at -70°C over time, to determine the 1st order rate constant for the loss so the final data set can be corrected accordingly.

Results: 3-(a). The data to date is shown in the table below. The compounds seem to be stable. More data is needed – and will be obtained before a definitive statement can

TIME (d)	RvD1	RvE1	LX4	MARESIN	12-HETE
0	100	100	100	100	100
1	92 ± 4	96 ± 4	89 ± 4	94 ± 6	84 ± 7
2	104 ± 7	98 ± 4	99 ± 3	103 ± 3	84 ± 2
7	99 ± 3	95 ± 5	91 ± 5	95 ± 3	104 ± 12
30	104 ± 4	97 ± 2	75 ± 8	108 ± 3	99 ± 5

be made concerning whether we will or will not need to correct for storage time, but based on presently available data, it looks like this won't be necessary. The next data point is due to be collected in October 2011.

While our study was in progress, a paper by Maddipati was published. This paper examined the stability of a large number of eicosanoids and docosahexanoids – including most of those relevant to this study in a series of aqueous buffers at 4°C, 20 °C and 37 °C (1). There was some (~10%) loss over 24 h at room temperature. If losses are small at these temperatures, they should be negligible at -70°C. Nevertheless we would like to have our own confirmatory data, hence we will continue with our storage stability experiment.

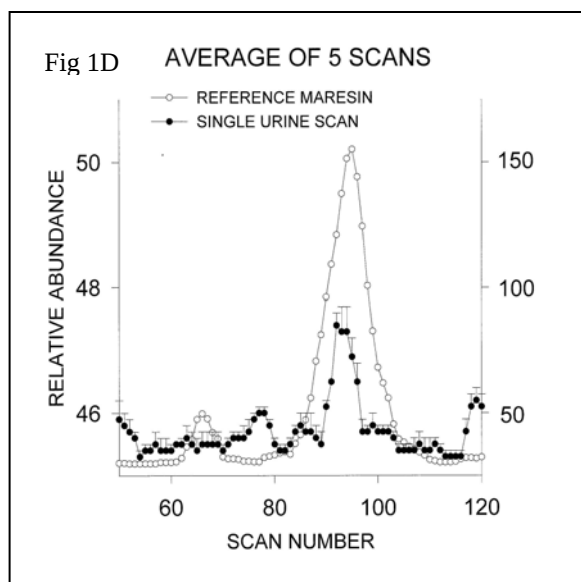
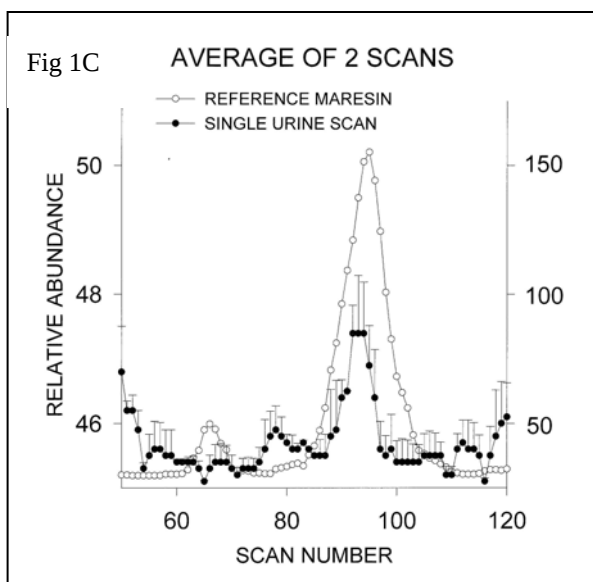
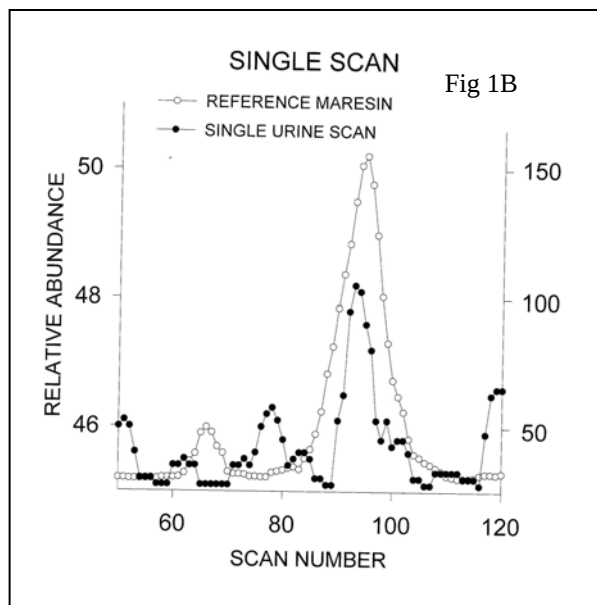
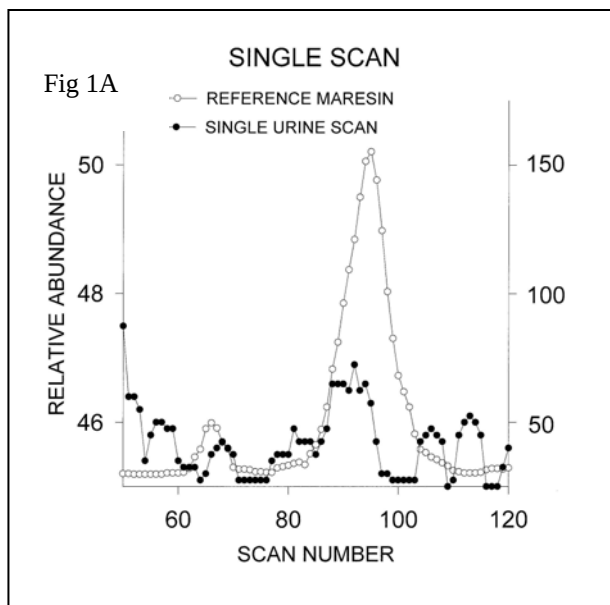
4: IMPROVEMENT IN ANALYTICAL SENSITIVITY

The results for objective #1 showed that, as expected concentrations of resolvins, Maresin (and neuroprotectin) were low. Reliable measurement would be greatly improved by reducing the noise in the mass spectra thereby making it easier to unambiguously identify and quantify the peaks of interest.

3-(a). Results: We believe we have developed an approach that increases the sensitivity and precision of all of the LC-MSMS assays by at least a factor of five. This is very important for the project. It will enable us to deliver far better data than original anticipated.

The basis of the method is to collect multiple scans for accurately defining peaks. Thus each sample is injected 5 consecutive times followed by standard blanks. The raw data (actual MRM abundances for each sampling point) are then converted to a CSV file and exported into Excel. The five spectra are then averaged and the Excel summaries exported to a chromatogram reconstruction/deconvolution program (Peakfit, SPSS, Chicago Ill.) and the area under the MRM curve integrated. The series of figures shows how this improves the quality of the data for Maresin.

Figures 1a and 1b show two consecutive matrix corrected chromatograms from the LC-MSMS. Note the very small abundances, the noise and the differences in peak shapes and abundances between two injections of the same sample. The background noise is because the instrument is running at a high electron multiplier voltage. The noise is too much to allow for an accurate peak area integration and calculation of a credible value for Maresin concentration. Figure 1c shows the average MRM spectrum of figures 1a and 1b. Note that a credible peak, albeit with large error bars results. Figure 1d shows the result if five consecutive injections are analyzed this way. Note that what cannot reliably be identified from a single injection is easily seen if five injections are averaged. Note the error bars. These are credible peaks. The calculated concentration of Maresin in the urine specimen is 0.42 ng/ml.



Task #4 Use the newly developed assays from task #3 to measuring the markers (isoprostane and its metabolite, LXA4, RvD1, RvE1 and Protectin), in the urines collected as part of the clinical trial. We anticipate 66 (placebo-treated) and 66

(DHA-treated) subjects; initially two urines pre-treatment and two at the end of the treatment phase will be analyzed for each metabolite. This task will be started one year after the start of patient sample collection. We anticipate having to do multiple LC-MSMS injections because while sample preparation will be common, LC conditions for resolution may well be different. We aim to do the analyses in not more than three batches, resolving several but not all of the compounds in each run (T.P. Stein and technician).

This task will not begin until subject recruitment, enrollment and treatment have been completed.

Task 5

Data will be collected and analyzed (04 year as per the current SOW, S Buyske).

This task will not begin until subject recruitment, enrollment, treatment and analysis have been completed.

Task 6

Manuscripts prepared and submitted for publication (04 year as per the current SOW, all investigators)

This task will not begin until subject data analysis has been completed.

Key Research Accomplishments

We have established a set of LC-MSMS conditions appropriate for testing the presence of LXA4, RvD1, RvE1 and Maresin in urine.

Reportable Outcomes:

There are no reportable outcomes at this time.

Conclusion:

We have made good progress with the necessary organic syntheses (task #2). (1) As stated in the prior annual progress report we expected these tasks to be completed within 15 months. This included making the corresponding deuterated analogs. Even though most of these are new and original syntheses, we are on schedule and all the necessary synthesis have been completed. The synthesis need now will be to replenish stocks of standards. (2) We now have a LC-MSMS analytical program to investigate which of the bioactive mediators can be detected in urine of the study patients and which are altered with fish oil supplementation.

In summary, paradoxically the delay in obtaining IRB approval has served us very well. Had we started on time last year we would have been locked into trying to modify state of the art analytical methodology (LC-MSMS based) to our older GC-MS

methodology with no guarantee of success. Thanks to the generosity of the Dean of UMDNJ-SOM we now have state of the art instrumentation and this greatly increases the probability of success for this project. Additionally we have submitted a grant with Dr. Stein as the PI and with Drs. Ming and Johnson as Co-PIs to NIH entitled: Glucuronidation and Autism.

We will attend the Autism NJ Jersey State Autism conference. On October 14th 2011.

We will present a poster:

T.P. Stein PhD, X. Ming MD, PhD, M.D. Schluter BS, A. Rodriguez PhD, B.W. Spur PhD and G. Lambert MD.

POLYUNSATURATED FATTY ACIDS METABOLISM IN AUTISM:

This will be done in a concerted effort with others from the initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730 to help make others aware of our work and our study and to increase interested and recruitment.

Dr. Novotny will be making a presentation titled “Alternative Treatments in Autism” at the conference. In her talk she will be speaking of the use of Omega-3 fatty acids in autism.

We will have a booth to distribute our recruitment materials and Dr's. Ming and Wasiulla and Mr. Stenroos will attend the conference with the goal of informing autism families, doctors, representatives of autism schools and autism centers/departments of hospitals as well as researchers of our study and making contact with potential recruitment sources.

Please see partnering project W81XWH-08-1-0730, Task #2 for more detail.

Appendices:

There are no appendices.

REFERENCES

1. Maddipati, KR and Zhou, S-L. Stability and analysis of eicosanoids and docosanoids in tissue culture media. Prostaglandins & other Lipid Mediators 94: 59–72, (2011).