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Fish oil in lupus nephritis: Clinical findings and methodological implications

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Fish oil in lupus nephritis: Clinical findings and methodological implications. Our objective was to determine the effects of fish oil on renal function, symptoms, and serum lipids in patients with lupus nephritis. A double-blind, randomized crossover trial of fish oil versus placebo (olive oil) was done on 26 patients with confirmed systemic lupus; 21 completed the study. Intervention was fish oil or placebo, 15 g/day, for one year followed by a 10 week wash-out period, and then the reverse treatment for one year. At baseline and six month intervals, we measured platelet membrane fatty acids, indices of renal function, a disease activity index, serum lipid levels, blood pressure, serum viscosity and red cell flexibility. We found that platelet membrane phospholipids were uniformly affected by fish oil supplementation ($P < 0.001$) but with significant carry-over effects despite a 10 week wash-out period. Glomerular filtration rate and serum creatinine were not affected. A non-significant reduction in mean (SE) 24-hour proteinuria occurred, from 1424.1 mg (442.7) on placebo to 896.7 mg (352.2) on fish oil ($P = 0.21$). Fish oil lowered serum triglycerides from 1.89 (0.25) mmol/liter to 1.02 (0.11) mmol/liter ($P = 0.004$). VLDL cholesterol decreased markedly whether patients initially received fish oil or placebo ($P = 0.004$). The size of the reduction was affected by the order of treatment ($P = 0.03$), but parallel comparisons were significant before the crossover ($P = 0.0006$). With the possible exception of bleeding time, no other treatment effects were shown with fish oil. However, treatment order effects were seen in urinary IgG excretion ($P = 0.03$), whole blood viscosity ($P < 0.0001$), red cell flexibility ($P = 0.004$), and bleeding time ($P = 0.06$). In conclusion, one year of dietary supplementation with fish oil in patients with stable lupus nephritis did not improve renal function or reduce disease activity, but did alter some lipid parameters. Hitherto unreported carry-over effects and treatment order effects caused by the olive oil created a risk of type II error, and bear methodologic consideration in the design of future studies.

With improvements in long-term prognosis, patients with systemic lupus erythematosus (SLE) now manifest a clinical course of early inflammatory and late atherosclerotic vascular events [1–9]. These observations have attracted our interest in fish oil as a form of nutritional intervention in human systemic lupus erythematosus. Fish oil and its principal constituents, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), possess both anti-inflammatory and anti-atherosclerotic properties [10]. Omega-3 fatty acids inhibit inflammatory prostaglandin and leukotriene production by displacing the arachidonic acid (AA) substrate from cell membranes and competing with the AA for cyclooxygenase and lipoxygenase enzymes [11–13]. This competition results in a shift towards the production of non-inflammatory series-3 prostaglandins and series-5 leukotrienes [12, 13]. Fish oil supplementation has been found to induce clinical responses in inflammatory diseases including rheumatoid arthritis, psoriasis and ulcerative colitis [14–18]. Fish oil supplementation has also been implicated in the lowering of lipid levels and a reduction in atherosclerotic vascular events in humans [19–24]. This dual action of fish oil has the potential to alter the bimodal morbidity and mortality pattern related to inflammatory and atherosclerotic events in patients with lupus nephritis.

In three different experimental murine models of lupus (NZB/W, BXSB, MRL/1PR) dietary fish oil reduces proteinuria and renal morphologic injury [25–29]. In the NZB/W and the BXSB mouse it also decreases mortality [25–27]. The anti-inflammatory and anti-atherosclerotic potential of fish oil coupled with the findings in the murine experiments prompted us to test the effects of fish oil supplementation in patients with lupus nephritis. Our previous dosing study of fish oil (MaxEPA) in lupus nephritis patients indicated that both low (6 g/day) and higher doses (18 g/day) were well tolerated and inhibited inflammatory mechanisms, but only the higher dose corrected dyslipidemias [30]. We now report a prospective placebo-controlled, single center, double-blind crossover study of the effects of dietary fish oil supplementation on renal function, dyslipidemia, clinical disease activity and immunologic markers of inflammation in patients with lupus nephritis.

Methods

Patients

The study was approved by the Review Board for Health Sciences, University of Western Ontario. Forty-three patients with the diagnosis of lupus nephritis attending nephrology clinics at the University of Western Ontario were invited to participate in the study. Twenty-six patients consented to

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Table 1. Patients completing 2 year + 10 week double-blind cross-over study

Patient	Sex	Age	ANA	Renal biopsy[a]	Proteinuria[b] *mg/24 hr*
Group 1: Fish oil 1st year					
1	♀	44	+	DPGN	106
2	♀	37	+	No biopsy	190
3	♀	49	+	DPGN	461
4	♀	22	+	No biopsy	7456
5	♀	47	+	DPGN	55
6	♀	55	+	FPGN	1465
7	♀	37	+	No biopsy	148
8	♂	53	+	DPGN	70
9	♀	27	+	DPGN	2501
10	♀	35	+	DPGN	1489
11	♂	66	+	DPGN	1910
12	♀	35	+	DPGN	1802
13	♀	35	+	FPGN	130
Group 2: Olive oil 1st year					
1	♀	30	+	DPGN	451
2	♀	37	+	DPGN	630
3	♀	30	+	DPGN	133
4	♀	38	+	No biopsy	45
5	♂	32	+	No biopsy	43
6	♀	38	+	No biopsy	262
7	♂	35	+	DPGN	5596
8	♀	28	+	No biopsy	6541

[a] Biopsy classification: DPGN, diffuse proliferative; FPGN, focal proliferative.
[b] Proteinuria at the onset of the study

participate and 21 completed the study. The other five indicated either a loss of interest or relocation as their reason for dropping out. Thirteen of the 21 patients began the trial on fish oil (hereafter Group 1) and completed it on placebo (olive oil supplement). The remaining eight patients (Group 2) were given the treatments in reverse order.

The characteristics of the patients in the two groups are recorded in Table 1. The mean (SE) age of the study population was 38.6 (2.3) years. All patients met a minimum of four of the ARA criteria for the diagnosis of SLE and all were ANA positive [31]. Nephritis associated with SLE was diagnosed by the presence of proteinuria of $\geq$ 500 mg/24 hr, or an active urinary sediment defined by the presence of one or more red cell cast, or $\geq$ 10 red cells/high power field (400 $\times$ magnification), 75% of which were dysmorphic. Fourteen patients had undergone previous renal biopsy, 12 indicating diffuse proliferative glomerulonephritis and two indicating focal proliferative glomerulonephritis (Table 1). At the onset of the study most patients were in a stable condition with 13 showing proteinuria of greater than 150 mg/24 hr, and 20 showing active urinary sediments.

Trial design

The design was a standard double-blind, randomized cross-over comparison, where fish oil (MaxEPA) was paired with placebo (olive oil) [32]. After giving informed consent patients were randomized to receive fish oil (Group 1) or placebo (Group 2) for one year. This was followed by a 10 week wash-out period during which no capsules were provided. Patients then crossed over to begin the second one year period of study on the other dietary supplement. Patients were asked to take five fish oil or placebo capsules three times per day. The 15 MaxEPA capsules contained 2.7 grams of EPA and 1.7 grams of DHA. Both fish oil and placebo capsules provided equal calories (157.5 Kcals). Patients were asked to maintain their usual diet throughout the study. Both patients and physicians were blinded to the treatment status.

Medication

Patients receiving non-steroidal anti-inflammatory therapy or cytotoxics received the same dose throughout the trial. Prednisone dosages were adjusted by the attending physician (WFC) who was blinded to the dietary supplementation period. Dosage changes were based on clinical responses and/or biochemical and serologic changes.

Measurements

At initiation and at six month intervals patients presented after an overnight fast and underwent clinical, blood and urine testing, including analysis of recent 24-hour urine collections for protein estimation. Blood was withdrawn from the subjects for determination of routine hematology, biochemistry and immunology analysis as well as platelet membrane phospholipids, serum lipids, whole blood viscosity, and red cell flexibility measurements. A urinalysis was performed on fresh urine collected at each visit. The patients also underwent a three-hour Tc-DTPA GFR study at each visit. During the study period subjects were assessed by the same physician, blinded to the dietary supplementation, who scored the SLE Disease Activity Index (SLEDAI) (Appendix 1) [33] and carried out blood pressure determinations in a recumbent position with Korotkoff's fifth sound as the diastolic blood pressure cut-off.

Patient compliance

This was assessed by capsule counts and by the platelet membrane n-3 (including EPA and DHA) versus n-6 fatty acid incorporation. Patients were given a surplus of capsules by the study pharmacist. The pill count was performed by the blinded research coordinator when patients returned at the six-month study intervals and received a new six month supply of capsules.

Platelet phospholipid fatty acid content

Platelets obtained from blood collected in EDTA, were washed with a phosphate buffer saline (PBS; 120 mmol sodium chloride, 10 mmol sodium phosphate and 13 mmol sodium citrate, pH 6.8) and then resuspended in Hanks buffered salt solution. One ml suspension (1×10^9 platelets) was used for lipid extraction by a procedure modified from that of Bligh and Dyer [34]. For this purpose, 3.5 ml methanol chloroform (2:1) was added and the tube was vortexed for 60 seconds. Finally, 1.75 ml distilled water was added and vortexed for 15 seconds. The tubes were then stored at −70°C until assayed [35]. Phospholipids from the lipid extracts were separated by thin-layer chromatography using silica Gel H as the stationary phase (Merck Co., Darmstadt, Germany) followed by development in heptane:isopropyl ether:acetic acid (60:40:3, vol/vol/vol). The bands corresponding to the different lipid fractions were sprayed with a solution of 2,7-dichlorofluorescein (Sigma Chemical Co., St. Louis, Missouri, USA) and visualized under ultraviolet light. The phospholipid band was scraped from the

plates, known amounts of monopentadecanoin (Nu-Check Prep Inc., Elysian, Minnesota, USA) added as the internal standard and samples transmethylated at 80°C with 6% H_2SO_4 in methanol (vol/vol). The resulting fatty acid methyl esters were identified by a comparison of their retention times to those of known fatty acid standards, using the Hewlett Packard, Model 5890A gas chromatograph, equipped with a DB225 megabore column (Chromatographic Specialties, Brockville, Ontario, Canada). The gas flow rates (ml/min) were 356 for air, 35 for hydrogen and 31 for nitrogen, and the flame ionization detection temperature was 250°C during these isothermal runs [36].

Renal function tests

Total urinary proteins were measured using sulfasalicylic acid precipitation. Glomerular filtration rate (GFR) was determined by injecting Tc-DTPA and constructing clearance curves based on three consecutive hourly blood samples [37]. Serum creatinine was measured by kinetic Jaffè chromogen reaction using Paramax Analytical System (Baxter, Irvine, California, USA). Urinary IgG was measured in aliquots of 24-hour urine collections by the ELISA technique using reagents from Nordic Immunological Laboratories (Cedarlane Labs, Hornby, Ontario, Canada).

Plasma lipid analysis

Plasma samples were obtained from blood collected in EDTA on ice. Total plasma cholesterol and plasma triglyceride levels were determined by enzymatic reagents obtained from Boehringer-Mannheim, Montreal, Quebec, Canada (cholesterol: CHOD-PAP; triglycerides: without free glycerol). The HDL cholesterol was determined enzymatically following precipitation of plasma with dextran sulphate-magnesium chloride as described by Warnick, Benderson and Albers [38]. VLDL and LDL cholesterols were calculated as described by Freidewald, Levy and Fredrickson [39].

Red cell flexibility

Heparinized blood was centrifuged for two minutes in a Clay Adams Autocrit centrifuge (Clay Adams Co., Parsippany, New Jersey, USA). Voltage supplied to the centrifuge was adjusted with a Powerstat variable transformer (Speer Electric Co., Bristol, Connecticut, USA) to achieve 200 g. Red cell flexibility (RCF) was measured as a percentage of cell packing/min as outlined by Sirs [40].

Whole blood viscosity (WBV)

The viscosity of heparinized whole blood relative to water was measured using a white cell pipette at room temperature according to the method of Wright and Jenkins [41].

Bleeding time

Bleeding time (expressed in minutes) was performed using the Simplate II device (Organon Teknika Corp., Durham, North Carolina, USA).

Immunology

Serum complement (C3 and C4) were measured by scattered light turbidity in a nephelometer (BNA Nephelometer, Behringwerke AG Diagnostica, Marburg, Germany). Antibodies to double-stranded DNA antibodies (ds-DNA binding activity) were measured in sera using the Farr ammonium sulphate precipitation technique. The data were transformed for analysis using the formula: (DNA value − 0.20)/(0.20 × 100), which was necessitated because our Immunology Laboratory could not measure levels below 0.20 (these are reported simply as <0.20).

Sample size

The planned sample size of 32 was based on feasibility and on the previous dosing study which showed a 25% relative increase in HDL levels over five weeks [30] and labeled it statistically significant with a much smaller number of patients. Using the observed means and standard deviations from baseline data in this study, and assuming a 25% increase in HDL levels, a two-tailed α of 0.05, and a β of 0.80, the N needed per group in a cross-over design was 13 [42]. Hence, before drop-outs, the study was adequately powered to detect and label significant the desired change in HDL. For other outcomes, no evidence-based sample size predictions could be made since there were no similar longitudinal or intervention studies in SLE.

Data analysis

Statistical analyses followed the suggestions of Armitage and Berry [43] and Fleiss [44]. A randomized cross-over trial is, in effect, two trials in one. An unpaired comparison is possible across groups according to whether subjects are on or off treatment, while within-patient paired comparisons are possible across periods (before and after cross-over). For any given patient in this trial we combine these two elements by creating paired change scores for the corresponding times on or off treatment (such as baseline matched to washout, 6 month treatment matched to 6 month placebo, and 12 month treatment matched to 12 month placebo). Mean change scores can then be compared on an unpaired group basis according to whether patients first received treatment or placebo. This analytical approach generates four P values:

(1) Treatment effects represent the comparison of values on treatment versus placebo, without regard for the order in which each was given.

(2) Time effects capture the difference between the two periods of the study and reflect the fact that patients may improve or deteriorate during the course of investigation. This entails simply comparing the overall level of measures before cross-over (that is, when some patients are on treatment and others on placebo) to those occurring later (that is, when nothing should have changed except that treatment and placebo assignments are reversed).

(3) Carry-over effects can result from an insufficient wash-out period at time of cross-over. This simply entails comparing the baseline and washout measures, taking specific account of whether the patient had been on treatment or placebo prior to the washout period. When a carry-over effect was found, we controlled for it using the baseline and washout measures as covariates [43]. The placebo in carry-over trials is usually assumed to be inert, hence the test is oriented to detecting differential carry-over effects. In this study the placebo turned out to have its own biological activity, which at times was similar to the treatment effect. Therefore we may have at times failed to designate a carry-over effect as statistically significant when both placebo (olive oil) and fish oil had carryover effects

Table 2. Fatty acid contents of platelet membrane phospholipids (mol%) and serum lipids (mmol/L)

Variable	Baseline	6 Month treatment	12 Month treatment	Wash-out period	6 Month placebo	12 Month placebo
Group 1	17.20	11.83	10.71	13.87	14.18	17.90
	(0.83)	(0.76)	(0.44)	(0.85)	(1.10)	(0.94)
AA						
	15.56	10.14	11.70	15.54	14.39	14.53
Group 2	(1.32)	(1.30)	(1.70)	(1.85)	(0.78)	(1.14)
EPA	0.34	2.63	2.49	0.59	0.38	0.39
	(0.04)	(0.20)	(0.20)	(0.07)	(0.03)	(0.04)
DHA	1.03	2.01	2.05	1.41	1.07	1.13
	(0.08)	(0.16)	(0.18)	(0.12)	(0.09)	(0.09)
N3/N6	0.12	0.41	0.45	0.19	0.13	0.13
	(0.01)	(0.03)	(0.04)	(0.02)	(0.01)	(0.01)
EPA/AA	0.02	0.26	0.25	0.04	0.03	0.02
	(0.004)	(0.02)	(0.02)	(0.004)	(0.002)	(0.02)
DHA/AA	0.06	0.20	0.19	0.10	0.08	0.07
	(0.004)	(0.02)	(0.02)	(0.01)	(0.01)	(0.004)
Triglyceride	1.89	1.09	1.02	1.25	1.75	1.61
	(0.25)	(0.18)	(0.11)	(0.15)	(0.24)	(0.20)
Group 1	0.96	0.47	0.45	0.63	0.89	0.79
	(0.14)	(0.08)	(0.04)	(0.10)	(0.14)	(0.12)
VLDL						
	0.65	0.66	0.64	0.71	0.55	0.49
Group 2	(0.15)	(0.17)	(0.14)	(0.20)	(0.18)	(0.12)

Data are mean values on or off treatment with standard errors of means in parenthesis.

of similar magnitude. This could have masked the main treatment effects in some instances, and led to order effects (see below) in others.

(4) Lastly, it is possible that the magnitude of the fish oil effect might be significantly altered according to whether patients received treatment first or placebo first. These order effects stem from a variety of interactions among treatment, time, carry-over, and other unmeasured effects. Where order effects are found, it is prudent to report both the two-period treatment effect, and a separate pre-cross-over or first period effect. The latter provides a parallel comparison of treatment and placebo in the absence of any confounding order influences.

Results

Drug compliance

An average of 14 capsules were taken daily with the range from 11 to 15. Good compliance was also suggested by the marked changes in platelet membrane phospholipids (Table 2).

Tabulation of outcome measures

Tables 3 and 4 provide *P* values for treatment, order, time and carry-over effects for the key outcome measures. Actual measured values [mean (SE)] are summarized in Tables 2 and 5, combining equivalent time periods for the two groups. Where order effects were potentially significant ($P < 0.10$) data are broken down into separate groups, and a *P* value reported for the parallel groups comparison of Group 1 on treatment versus Group 2 on placebo.

Serum lipids and platelet membrane phospholipids

Among lipid measures, only serum triglyceride and VLDL cholesterol showed a significant decrease (Table 2). Neither LDL nor HDL cholesterols were significantly affected. Carry-over effects were found in all platelet membrane phospholipid fatty acids except AA (Table 3), but with or without adjustment for carry-over influences, fish oil had a significant effect on platelet membrane phospholipid fatty acid contents. Order effects were found for AA and VLDL cholesterol (Table 2), suggesting that olive oil itself affected VLDL and AA levels. However, a parallel group comparison for VLDL and AA confirmed the presence of treatment effects prior to cross-over.

Table 3. Analysis of treatment and other effects: Platelet phospholipid fatty acid content and serum lipids

Variable	Treatment effect	Time effect	Order effect	Carry-over effect
	P values			
N3/N6	<0.0001	0.12	0.30	0.02
DHA	0.0004	0.16	0.85	0.01
EPA	<0.0001	0.49	0.61	0.004
AA[a]	0.0011	0.09	0.01	0.64
DHA/AA	<0.0001	0.04	0.85	0.02
EPA/AA	<0.0001	0.23	0.89	0.004
Triglyceride	0.04	0.10	0.44	0.71
VLDL-cholesterol[a]	0.004	0.10	0.03	0.54

[a] Pre-cross-over effects: AA, $P = 0.003$; VLDL, $P = 0.0006$

The level of baseline proteinuria was correlated to the absolute magnitude of the treatment-induced changes in triglycerides and VLDL. Changes were calculated as the 10-week treatment value versus the baseline for the corresponding period. Correlations were -0.595 ($P = 0.004$) and -0.602 ($P = 0.004$), respectively.

Renal function

There were no significant changes in renal function measures (Tables 4 and 5). However, the mean (SE) 24-hour urinary protein excretion over one year of fish oil supplementation was

Table 4. Analysis of treatment and other effects: Renal, rheologic, and immunologic measures

Variable	Treatment effect	Time effect	Order effect	Carry-over effect
	P values			
GFR[a]	0.48	0.25	0.05	0.70
Inverse serum creatinine (log)	0.46	0.86	0.97	0.90
24 Hr urinary protein (log)	0.22	0.92	0.50	0.92
Urinary IgG (log)[a]	0.12	0.32	0.01	0.94
WBV[a]	0.59	0.64	<0.0001	0.68
RCF[a]	0.75	0.66	0.004	0.74
Bleed time[a]	0.01	0.02	0.06	0.80
C3	0.94	0.0002	0.90	0.44
C4	0.27	0.08	0.56	0.91
Anti-ds-DNA	0.71	0.25	0.35	0.92

[a] Pre-crossover effects are: GFR, $P = 0.60$; IgG, $P = 0.47$; WBV, $P = 0.59$; RCF, $P = 0.14$; Bleed time, $P = 0.58$

reduced from 1424.1 mg (442.7) to 896.7 mg (352.2), $P = 0.21$. When change in proteinuria was defined categorically as a change greater than 100 mg of protein excretion per 24 hours, nine patients showed decreased proteinuria, but three had increased levels and nine patients exhibited no change on fish oil (Tables 5 and 6). Urinary IgG showed an order effect ($P = 0.02$ for raw data, 0.03 with a log transform to correct for skew) but no treatment effect (Table 4). GFR showed an order effect ($P = 0.05$) but no treatment effect (Tables 4 and 7). Serum creatinine remained unchanged throughout the study. For IgG and GFR, where order effects were found, no treatment effects were detected upon analyzing pre-cross-over data in isolation.

Clinical measures

The mean SLEDAI scores as well as mean daily steroid doses (Table 8) were not altered by either fish oil or olive oil supplements. There was also no significant effect on mean (SE) blood pressure, which fell on fish oil from 101 [3] mm Hg to 96 [2] mm Hg.

Rheologic measurements

Whole blood viscosity was reduced after one-year of fish oil treatment in Group 1 patients from 4.6 (0.19) to 2.9 (0.11), but was unaltered in Group 2 patients, 2.8 (0.11) to 2.9 (0.11). This led to an insignificant overall treatment effect but a marked order effect (Tables 4 and 5). Order effects ($P = 0.004$) were also seen for red cell flexibility, without any fish oil effect. Fish oil appeared more effective than the placebo in increasing bleeding time (treatment effect $P = 0.01$). However, there were order and time effects, and the pre-crossover analysis showed no significant treatment effects ($P = 0.58$), rendering the results difficult to interpret.

Immunologic markers

The individual data for C3, C4 and ds-DNA antibodies are presented in Tables 9, 10 and 11 respectively. The C3 levels, and to a much lesser extent C4 levels, show time effects (Table 4), suggestive of a cumulative effect from fish oil and olive oil, where both had immunomodulatory effects. There were no effects for ds-DNA. The analysis was repeated analyzing ds-DNA as a categorical outcome (levels <0.20 or above 0.20), with similar results.

Discussion

Lupus nephritis patients suffer from inflammatory and atherosclerotic vascular events. The steroid and cytotoxic treatment of lupus nephritis is based on clinical observations, historically controlled, or retrospectively pooled analyses of controlled studies [2, 4, 45]. The success and failure of these therapies prompted us to assess fish oil dietary supplementation since it has been noted to exert both an anti-inflammatory and anti-atherosclerotic effect.

The present study is a single center trial. The double-blind cross-over design reduces the inherent individual variability of lupus nephritis by comparing each subject with himself or herself during each dietary supplementation period. However, the validity of certain conclusions of this study is threatened by the fact that the 10-week washout period does not completely prevent a carry-over effect as shown on comparing platelet membrane phospholipid levels prior to fish oil and following washout. Furthermore, the olive oil placebo exerts an effect on plasma viscosity, red cell flexibility, AA, VLDL cholesterol, C3 and C4 in a direction that appears similar to fish oil. No statistical methodology can fairly adjust for both effects concurrently.

Olive oil was used as a placebo for fish oil because its constituents are similar to, but in much lower concentration than, the typical constituents of a North American diet. Indeed, olive oil has been used almost exclusively as the placebo in fish oil studies [14–18, 46]. All recent cross-over double-blind studies with fish oil have used olive oil as a placebo and most have included a much shorter washout period with equal or greater doses of fish oil. Although some of these studies addressed issues arising from the study design, none performed and/or reported a full analysis of the carry-over or order effects. We presume that these studies suffered from both carry-over and unanticipated olive oil effects as did ours [14–16, 18].

In all three lupus nephritis mouse models dietary supplementation with fish oil in the form of Menhaden oil or MaxEPA resulted in a reduction in proteinuria and improvement in renal morphology [25–29]. The impact on human renal function has been disappointing. Westberg and colleagues, for example, have reported a double-blind cross-over study of MaxEPA and placebo for six months in a mixed renal and non-renal population of patients with systemic lupus erythematosus [46]. Although proteinuria was not a frequent feature of their seventeen patients, GFRs were not significantly changed by fish oil. While we did not assess renal morphology, proteinuria and isotope GFR were measured with no statistically significant changes detected. However, statistical power limitations merit mention. Using the baseline data from the trial, and assuming that we wished to detect and label significant a 25% treatment/placebo difference in GFR, 15 patients per arm were needed for 80% power. The present sample gives us about 65% power to detect a 25% change in GFR [42]. More importantly, we observed a 37% reduction in mean levels of proteinuria, but the extreme variability in this outcome meant that the observed difference was compatible with the play of chance alone. Actual *post hoc* power was under 20% for this outcome, underscoring the need for a much larger sample size or more homogeneous patient

Table 5. Mean values by period: Renal and other measures

Variable	Baseline	6 Month treatment	12 Month treatment	Wash-out period	6 Month placebo	12 Month placebo
Bleeding time *min*						
Group 1	5.40	5.20	5.60	6.20	6.20	6.0
	(0.42)	(0.33)	(0.39)	(0.39)	(0.50)	(0.53)
	7.20	7.80	7.60	5.40	5.90	5.40
Group 2	(0.67)	(0.60)	(0.46)	(0.67)	(0.67)	(0.57)
Red cell flexibility						
Group 1	15.6	14.0	18.6	18.7	18.6	17.7
	(1.28)	(1.74)	(1.94)	(1.41)	(1.69)	(1.97)
	18.0	16.8	16.5	16.9	8.7	16.4
Group 2	(2.09)	(2.12)	(1.52)	(1.84)	(1.63)	(3.01)
Whole blood viscosity						
Group 1	4.60	4.80	2.90	2.70	3.0	2.90
	(0.19)	(0.28)	(0.09)	(0.11)	(0.11)	(0.11)
	2.80	2.60	2.90	4.30	4.00	2.90
Group 2	(0.11)	(0.14)	(0.11)	(0.18)	(0.28)	(0.11)
GFR *ml/min/1.73 m²*						
Group 1	75.5	71.4	74.7	73.3	70.9	69.2
	(10.1)	(9.4)	(9.4)	(10.5)	(9.6)	(9.8)
	79.6	72.4	78.1	80.8	78.8	77.6
Group 2	(8.6)	(10.5)	(9.2)	(11.7)	(10.5)	(10.8)
Serum creatinine *μmol/ liter*	88.52	92.14	94.48	92.29	95.62	92.57
	(7.11)	(7.71)	(7.39)	(6.98)	(8.43)	(8.62)
Urinary protein *mg/24 hr*	1424.14	974.95	896.71	1270.90	1162.5	1547.95
	(442.67)	(321.52)	(352.21)	(447.17)	(364.20)	(466.77)
Urinary IgG *μg/min*						
Group 1	9.42	7.67	6.66	8.57	15.04	8.79
	(4.39)	(2.11)	(1.58)	(2.10)	(3.66)	(2.61)
	8.68	6.12	5.48	6.55	6.06	10.59
Group 2	(1.81)	(2.13)	(1.57)	(2.73)	(2.33)	(2.73)

Data are means with standard errors of means in parenthesis.

Table 6. Urinary protein *mg/24 hr*

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	106.00	203.00	193.00	138.00	266.00	430.00
2	190.00	39.00	94.00	105.00	1072.00	3231.00
3	461.00	72.00	61.00	121.00	107.00	122.00
4	7456.00	693.00	1023.00	955.00	396.00	1177.00
5	55.00	31.00	47.00	16.00	25.00	51.00
6	1465.00	1329.00	881.00	1000.00	2038.00	3254.00
7	148.00	42.00	165.00	100.00	22.00	45.00
8	70.00	398.00	701.00	516.00	543.00	1062.00
9	2501.00	5956.00	5453.00	4900.00	4359.00	294.00
10	1489.00	1726.00	204.00	106.00	406.00	80.00
11	1910.00	747.00	963.00	836.00	1177.00	2070.00
12	1802.00	1451.00	886.00	4016.00	6437.00	8137.00
13	130.00	424.00	89.00	179.00	186.00	53.00
Mean	1367.92	1008.54	827.69	999.08	1310.31	1538.92
SE	561.80	443.05	400.81	440.21	541.12	639.24
Geometric mean	490.96	352.25	325.37	323.88	432.16	452.37

Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	451.00	751.00	1180.00	1042.00	375.00	544.00
2	630.00	509.00	1576.00	663.00	473.00	238.00
3	133.00	48.00	161.00	209.00	136.00	320.00
4	45.00	22.00	90.00	46.00	61.00	65.00
5	43.00	11.00	34.00	33.00	19.00	37.00
6	262.00	541.00	380.00	369.00	98.00	432.00
7	5596.00	2697.00	3452.00	3850.00	3150.00	596.00
8	6541.00	2800.00	5609.00	5912.00	3051.00	5839.00
Mean	1712.63	922.38	1560.25	1515.50	920.38	1008.88
SE	967.07	414.44	713.75	776.72	483.90	700.81
Geometric mean	403.40	255.46	535.29	455.12	267.65	332.61

Table 7. GFR *ml/min/1.73 m²*

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	71	73	72	75	69	77
2	121	111	100	94	101	91
3	121	104	104	105	100	88
4	29	23	32	28	25	33
5	137	137	141	141	122	124
6	43	40	36	35	47	45
7	68	48	86	115	94	94
8	85	80	85	68	74	74
9	36	43	37	38	27	23
10	40	35	32	30	34	33
11	85	66	66	68	69	62
12	43	69	73	41	38	28
13	102	99	107	114	121	128
Mean	75.46	71.38	74.69	73.23	70.87	69.23
SE	10.11	9.40	9.40	10.47	9.63	9.82
Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	61	71	60	54	50	58
2	113	128	116	109	108	102
3	50	38	28	36	22	42
4	102	106	97	109	94	90
5	90	90	93	107	90	94
6	48	47	41	35	45	43
7	95	81	92	98	90	97
8	78	69	94	98	80	99
Mean	79.63	78.75	77.63	80.75	72.38	78.13
SE	8.64	10.45	10.91	11.71	10.52	9.16

Table 8. Prednisone dose *mg/day*

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	10/5	10/0	5	5	10	5
2	10	10/0	0	0	0	5
3	10	20	10	10	10	10
4	40	20	15	15	15	15
5	5	5	5	5	5	5
6	0	0	0	0	0	0
7	20	10	10	15	15	10
8	10	10	10	15	15	10
9	10	10	10	10	15	5
10	10	10	10	10/7.5	5	7.5
11	10	10	10	10	7.5	7.5
12	10/0[a]	10	10	10	10/20	10
13	10	5	5	5	5	0
Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	10	10/0	10	10	10	10
2	10	10/0	7.5	7.5	7.5	10
3	10	10/0	10/0	10	7.5	10/5
4	0	0	0	0	0	0
5	30	10	10	10	10	10
6	15	15/12.5	12.5	12.5	12.5	10
7	10	0	10	10	10	10
8	15	20	5	5	5/7.5	5

Note: 10/0 etc. describes alternate day therapy with 10 mg taken one day alternating with 0 mg the next.
[a] Patient had 2 weeks of 30 mg/day then returned to 10/0

Table 9. C3 *mg/liter*

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	947	807	530	498	586	495
2	583	463	421	389	364	413
3	549	346	400	426	581	560
4	1490	893	544	534	517	475
5	1080	881	630	666	666	631
6	1500	1120	797	753	877	766
7	897	737	695	651	579	558
8	712	478	494	424	487	557
9	1250	807	591	599	805	621
10	1080	793	649	589	618	595
11	1400	1200	941	833	982	866
12	724	1020	504	407	324	396
13	916	675	487	586	606	501
Mean	1010	786	591	566	615	572
SE	91	70	42	38	52	36
Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	777	611	530	453	451	511
2	773	621	524	486	530	650
3	650	583	395	391	313	392
4	1110	873	661	715	743	697
5	928	671	606	552	515	514
6	1020	847	638	557	524	614
7	1250	667	776	766	693	851
8	1120	816	992	872	711	861
Mean	954	711	640	599	560	636
SE	73	41	64	59	52	58

Table 10. C4 *mg/liter*

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	342	180	110	127	128	123
2	212	122	117	122	118	108
3	202	75	96	84	137	131
4	998	370	174	178	143	148
5	280	180	163	180	173	154
6	623	224	230	249	280	218
7	183	190	195	193	158	128
8	280	125	226	185	206	251
9	478	281	236	204	283	258
10	423	204	231	220	254	234
11	712	345	351	342	352	341
12	251	245	177	112	123	167
13	242	145	121	159	144	134
Mean	402	207	187	181	192	184
SE	68	24	19	19	21	19
Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	341	233	258	210	191	217
2	177	129	125	154	129	143
3	274	184	101	124	78	123
4	422	323	294	304	347	310
5	212	246	96	67	52	86
6	489	282	255	178	215	285
7	384	173	240	267	226	297
8	321	182	262	204	155	217
Mean	328	219	204	189	174	210
SE	37	23	29	27	33	30

Table 11. ds-DNA binding

Patient # Group 1	1st Year fish oil			2nd Year placebo		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	<0.20	<0.20	<0.20	0.24	<0.20	<0.20
2	0.27	0.24	0.48	0.57	0.45	0.42
3	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
4	<0.20	0.28	0.48	0.40	0.22	0.21
5	<0.20	0.26	0.23	0.31	0.26	0.47
6	0.21	0.22	<0.20	<0.20	<0.20	<0.20
7	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
8	0.27	0.23	0.28	0.28	<0.20	<0.20
9	<0.20	<0.20	<0.20	0.27	<0.20	<0.20
10	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
11	<0.20	0.21	0.22	<0.20	0.22	0.22
12	0.22	0.32	0.60	0.60	0.90	0.36
13	0.35	0.35	0.46	0.59	0.60	0.75
Patient # Group 2	1st Year placebo			2nd Year fish oil		
	Baseline	6 Months	12 Months	10 Week washout	6 Months	12 Months
1	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
2	0.32	0.35	0.32	0.40	<0.20	<0.20
3	0.37	0.36	0.47	0.45	0.77	0.66
4	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
5	0.37	<0.20	<0.20	<0.20	0.31	0.25
6	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
7	0.52	0.32	<0.20	<0.20	<0.20	<0.20
8	0.25	0.42	<0.20	0.21	0.29	<0.20

A value of <0.20 represents a negative result for ds-DNA binding activity.

populations in future studies assessing the effect of fish oil on proteinuria.

The absence of effect on clinical measures and symptoms in our study is also compatible with Westberg's previous study [46]. The SLEDAI is a symptom scale that is well validated [33, 47] but may not be the most appropriate measure to follow in patients with lupus nephritis as opposed to patients with predominantly extrarenal organ involvement. The scale provides a total of 105 points for the disease activity of which only 16 represent renal involvement (Appendix 1). This is a concern given that our patient population was relatively stable throughout the study and did not include severely active patients.

Fish oil dietary supplementation reduced triglycerides and VLDL cholesterol as previously noted in our dosing study [30]. Although we observed a rise in HDL cholesterol it was much smaller than in the short-term dosing study [30] and did not achieve significance despite our larger sample size. The reduction in triglycerides and VLDL cholesterol noted with fish oil has been observed in patients with and without hyperlipidemia [48–50]. Nestel et al have shown both *in vivo* and *in vitro* that the reduction in VLDL cholesterol associated with fish oil is due to a reduced synthesis of VLDL triglycerides and APO protein B [51, 52]. Findings from experimental animals, perfused liver systems and isolated liver cell preparations consistently indicate that fish oil reduces triglyceride synthesis by substrate diversion away from triglyceride formation [53]. The clinical significance of the lipid alterations observed in our study remains unknown.

There were slight reductions in mean blood pressure during the fish oil dietary supplementation, similar to those found in a larger series of patients with essential hypertension [54, 55]. The reduction in whole blood viscosity when ingesting fish oil has been noted by others and in our short-term dosing study [30, 55–57]. Neither fish oil nor olive oil led to a significant change in red cell flexibility, although fish oil did alter red cell flexibility in our previous short-term study [30]. A reduction in whole blood viscosity could potentially contribute to a decrease in glomerular capillary permeability and the resultant proteinuria [58].

In sum, this two-year double-blind cross-over study of stable lupus nephritis patients and Westberg's six-month double-blind cross-over study of lupus patients with mild nephritis have generated similar results [46]. In both studies GFR and clinical responses measured by different scales were not affected by fish oil or placebo. Moreover, in our lupus nephritis population the reduction in proteinuria during fish oil dietary supplementation did not achieve significance, but this may be attributable to variability in proteinuria levels and resultant low statistical power. Measures of dyslipidemia, specifically serum triglycerides and VLDL cholesterol, were significantly lowered with fish oil treatment. The modest improvements observed during fish oil dietary supplementation compared with placebo do not support a recommendation that fish oil dietary supplementation be considered a routine treatment for lupus nephritis patients. Given the placebo and carry-over effects noted in our analysis, we do recommend that any future cross-over trials assessing the effect of fish oil on dyslipidemias or renal function be performed without olive oil as a placebo and with a longer washout period. Better yet, we would urge that, where possible, cross-over designs be avoided given the complexity of the effects as shown here and the risk of similar problems in similar studies.

Lastly, we raise two general cautions about all studies of fish oil to date. First, the above-noted differential carry-over effects are important confounders in any study using a cross-over

design. Second, the general adoption of olive oil as a placebo is a potential source of type II error in both cross-over and parallel group designs. Studies showing beneficial effects of fish oil relative to olive oil placebo may therefore be underestimating the achievable benefits. Furthermore, in studies where fish oil is not found to be beneficial, re-examination of the findings or further study with a more physiologically inert placebo may be appropriate.

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Appendix. SLEDAI-DATA COLLECTION SHEET

(Enter weight in SLEDAI score column if descriptor present at the time of the visit or in the preceding 10 days)

Weight	SLEDAI Score	Descriptor	Definition
8		Seizure	Recent onset. Exclude metabolic, infectious or drug causes.
8		Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Includes: hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre disorganized or catatonic behavior. Exclude presence of uremia and offending drugs.
8		Organic brain syndrome	Altered mental function with impaired orientation, memory or other intellectual function with rapid onset, fluctuating clinical features. Such as any of the following: (a) clouding of consciousness with reduced capacity to focus and inability to sustain attention to environment. Plus at least 2 of (b) of perceptual disturbance; incoherent speech; insomnia or daytime drowsiness; increased or decreased psychomotor activity. (Exclude metabolic, infectious, drugs caused).
8		Visual	Retinal changes of SLE; any of cytoid bodies, retinal hemorrhages, serous exudate or hemorrhages in the choroid, optic neuritis. (Not due to hypertension or drugs or infection).
8		Cranial nerve	New onset of sensory or motor neuropathy involving the cranial nerve.
8		Lupus headache	Severe, persistent headache, may be migrainous, but must be non-responsive to narcotic analgesia.
8		CVA	New syndrome. Exclude arteriosclerosis.
8		Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, biopsy or angiogram proof of vasculitis.
4		Arthritis	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling or effusion).
4		Myositis	Proximal muscle aching/weakness, associated with elevated CPK/aldolase or EMG changes or a biopsy showing myositis.
4		Casts	Heme granular or RBC.
4		Hematuria	>5 RBC/hpf. Excluding other causes (stone, infection)
4		Proteinuria	>0.5 g/24 hours. New onset or recent increase of more than 0.5 g/24 hours.
4		Pyuria	>5 WBC/hpf. Exclude infection.
2		New rash	New onset or recurrence of inflammatory type rash.
2		Alopecia	New onset or recurrent. An abnormal patch of diffuse loss of hair.
2		Mucous membrane	New onset or recurrence of oral or nasal ulcerations.
2		Pleurisy	Pleuritic chest pain with pleural rub or effusion or pleural thickening.
2		Pericarditis	Pericardial pain with at least one of the following: rub, effusion, EKG, echo confirmation.
2		Low complement	Decreased any of CH50, C3, C4. Below the lower limit of normal for lab.
2		Increased DNA binding	>25% binding by Farr assay. Above normal range of lab value (eg. 25%)
1		Fever	>38° C After exclusion of infection.
1		Thrombocytopenia	<100,000 platelets.
1		Leukopenia	WBC < 3000 (not due to drugs)

Total SLEDAI Score: _____