NOAA TECHNICAL MEMORANDUM
NMFS-SEFSC-367

BIOMEDICAL TEST MATERIALS PROGRAM:
SYNOPSIS OF MEETING ON OMEGA-3 FATTY ACID RESEARCH
MAY 12, 1994
NATIONAL INSTITUTE OF HEALTH
BETHESDA, MARYLAND

by

PATRICIA A. FAIR and NORMAN SALEM, Jr.

U.S. Department of Commerce
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeastern Fisheries Science Center
Charleston Laboratory
P. O. Box 12607
Charleston, SC 29422-0607
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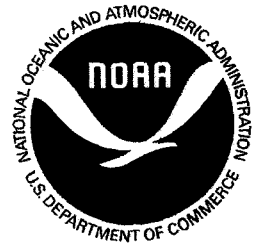

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U. S. DEPARTMENT OF COMMERCE
Ronald H. Brown, Secretary

NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
D. James Baker, Administrator

NATIONAL MARINE FISHERIES SERVICE
Rolland A. Schmitten, Assistant Administrator for Fisheries

MAY 1995

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Author's affiliations: Fair, P.A., NOAA, NMFS, Charleston Laboratory, Charleston, SC 29412; Salem, N., NIH, NIAAA, Rockville, MD 20852.

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EXECUTIVE SUMMARY

Patricia Fair, Ph.D. and Norman Salem, Jr., Ph.D.

The objective of the meeting was to identify the major information gaps in omega-3 fatty acid research and define impediments toward: a) definitive conclusions on the health effects of omega-3 fatty acids in each research area, and; b) clinical recommendations. Experts in several disciplines, including cardiovascular disease, thrombosis, cancer, metabolism, diabetes, inflammation, and neural development, discussed the status of omega-3 fatty acids and their effects in health and disease. Invited speakers and discussants were asked to critique each area and to indicate how the Biomedical Test Materials (BTM) Program can promote research in this area (meeting agenda attached). There were over 39 invited participants at the Fish Oil Test Materials Program Meeting (participant list attached). The meeting was chaired by Drs. Norman Salem (NIH) and Patricia Fair (NOAA).

- **INFLAMMATION RESEARCH** - Dr. Sperling indicated that several small clinical studies demonstrated the effects of low doses of omega-3 fatty acids in patients with rheumatoid arthritis, but that large, multi-study trials were needed to confirm data from these smaller trials. A meta-analysis of data from 395 patients in nine trials conducted by Drs. Sperling and Fortin, revealed significant improvements in the tender/painful joint count and morning stiffness with fish oil treatment after three months. A consensus recommendation was that a multi-center trial be conducted to evaluate the role of omega-3 fatty acids in the treatment of rheumatoid arthritis. Since high doses of fish oil are virtually without side effects, having minimal toxicity and weak non-steroidal effects, the higher doses of 6 g/day were indicated. It was thought that the clinical trials should be conducted first in promising areas and then mechanistic studies pursued. The use of omega-3 fatty acids as an adjunct to traditional drug therapy should be explored for synergistic effects.

- **CANCER RESEARCH** - Dr. Rose noted that there were several problem areas needing attention before clinical trials could be developed. Several issues identified were: 1) lack of information concerning dose-response inhibition of tumorigenesis, 2) potential adverse effects of lipid peroxidation products of fish oils, 3) specificity of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as effectors of tumor growth and metastasis suppression by fish oil, and; 4) translation of experimental studies to the clinic. While funding of a trial of omega-3 fatty acids as agents in the prevention of breast cancer may be difficult, serious consideration could be given to the use of omega-3 fatty acids as an adjuvant therapy regimen in a clinical trial. Clinical studies on the potential benefit of omega-3 fatty acids in cachexia should also be considered. There is a need for animal studies directed at understanding the modulatory influences of antioxidants, trace metals, vitamins, and their interactions with tumor responses to omega-3 fatty acids. Research is needed in which dose-response studies use concentrations of omega-3 fatty acids that are applicable to clinical settings. While the need for dose-response studies is well recognized, these types of studies are viewed as mundane and generally do not receive top priority for funding. Appropriate biomarkers of response to dietary omega-3 fatty acids need to be developed; ideally intermediate response

markers (such as thymidine index, 16- hydroxylated estrogen suppression in breast cancer). Dr. Karmali's comments regarding cancer research summarizes several promising results both in experimental and human studies on the chemopreventive actions of omega-3 fatty acids. Clinical studies on omega-3 fatty acids are promising in both colon and breast cancers. Additionally, the recent development of a biomarker for risk of breast cancer (adenosine diphosphate ribosyl transferase) should encourage a large-scale clinical trial. In cancer research, studies are needed concerning the transformation of normal cells, peroxidation, adjunct therapy, and colon cancer.

- **NEURAL DEVELOPMENT** - While efficacy data are available, the number of studies required for application to the general population is not sufficient. Many practical issues are ill-defined, including knowledge of the optimal ratios and amounts of various long chain polyunsaturates as well as the appropriate feeding interval for omega-3 fatty acids, the safety of omega-3 fatty acid products for preterms, and the effects of longer term feeding on growth. The difficulty expressed in such studies relate to the use of behavioral end points and the lack of standardization of formulas used in feeding trials. The importance of the maternal diet in infant studies as well as the variability of omega-3 fatty acid content in breast milk were stressed in evaluating infant studies. The importance of follow-up testing in older animals for evidence of permanent change was emphasized. Nevertheless, progress has been rapid in this area and it appears that it will lead to a public health benefit in the future.

- **INTERMEDIARY METABOLISM & DIABETES** - Dr. Bagdade listed factors that related to disparate findings in fish oil studies. These included: variable experimental conditions, substitution vs. supplement, placebo use, study duration, types of patients, types and doses of fish oil, content of cholesterol, saturated fat, and EPA/DHA ratios. The spectrum of anti-atherogenic properties due to omega-3 fatty acids are of sufficient magnitude to warrant a clinical trial in a population at risk, such as the diabetic population, particularly those with NIDDM (non-insulin dependent diabetes mellitus). It was concluded that omega-3 fatty acids have a number of anti-atherogenic effects and the effects on blood pressure and lipoprotein profiles could have long-term benefits. The ideal study population is patients with NIDDM since many of the risk factors are over-represented in this illness, and fish oils have the reported capacity to reverse many of these abnormalities. Dr. Bagdade strongly expressed a view with which discussants concurred that a controlled clinical trial be undertaken to assess whether omega-3 fatty acid treatment can reduce morbidity and mortality in this high risk population.

- **THROMBOSIS** - Dr. Harker's report on thrombosis indicated that there is substantial information on the cellular effects of omega-3 fatty acids related to vascular reactivity, atherogenesis and thrombosis. Dietary experiments substituting and/or supplementing omega-3 fatty acids have been performed in various animals often with conflicting results. Omega-3 fatty acids could alter vascular lesions at any number of steps. The design of clinical trials of omega-3 fatty acids in percutaneous transluminal coronary artery angioplasty (PTCA) must consider the dose (with large doses required to alter arachidonate metabolism), the timing of the initial dose prior to PTCA, the blindness of the study, and contain an angiographic

outcome to provide for an objective evaluation criteria. While no substantial benefit has been observed for omega-3 fatty acids after angioplasty, the studies do not answer the question of what anti-atherogenic benefits of omega-3 fatty acids may be obtained in man. Studies with experimental animals need to define 1) the dose relationship to omega-3 fatty acids, 2) purified EPA and DHA actions, and 3) molecular mechanisms. A pathophysiologic correlation must be demonstrated. Studies are needed on 1) sickle cell anemia and possible trials to define dose, 2) angiography to determine how to maintain patency and, 3) potential intervention. Researchers need to determine whether the methodology is sufficient and then if good surrogates can be measured.

- **HEART DISEASE & ARRHYTHMIA** - Despite the preponderance of information on the biochemical and physiologic effects of omega-3 fatty acids, adequate, placebo-controlled, double-blind clinical studies are lacking on their effects on atherosclerosis. Several retrospective dietary studies confirm the association with reduction of relative risk from coronary heart disease with ingestion of fish. Dr. Leaf's summary of 10 clinical studies on fish oil supplementation following PTCA found that while marginal benefits have been observed, the adequacy of the protocols used in these studies may be questioned. In order to maximize the effects of omega-3 fatty acids it is critical in such studies to: 1) have an adequate length of time for incorporation of omega-3 fatty acids in cellular membranes prior to PTCA and, 2) decrease dietary saturates and omega-6 fatty acids. Lessons should be learned from prior studies so that optimal clinical study designs can be used. While no positive effects were observed in some studies, it is important to note that there were also no complications from the doses of omega-3 fatty acids. Recently, animal studies confirm that ventricular arrhythmias can be prevented both *in vitro* and *in vivo* by omega-3 fatty acids. There is a need for purified fatty acids in the non-esterified form for use in such cardiovascular research. Obstacles for the use of omega-3 fatty acids in intravenous preparations are in part technical but also the concerns of the cardiovascular community are a factor. It was indicated that baroreceptor are also involved and must be studied. Evidence from animal models supports the benefits of omega-3 fatty acid treatment in sudden cardiac death caused by ventricular fibrillation. A definitive coronary heart disease (CHD) trial is needed to demonstrate the potential benefit of omega-3 fatty acids in patients with implantable cardiovertedefibrillators. Design criteria must consider the controls chosen and the effect of large body pools of fat that may be altered only after an extended dietary intervention. It is important to ascertain the effects of omega-6 and omega-3 fatty acids, as well as to distinguish EPA and DHA. A mortality trial is needed to demonstrate true correlations. There was much discussion as to what the most appropriate placebos should be. It was agreed that the placebo of choice depended upon the question being asked. The confounding effects of aspirin in CHD studies are problematic. Since omega-3 fatty acids have multi-factorial effects, an integrated pathological model is needed. Biological variability among species needs to be considered in relating results from animal studies.

- **BIOMEDICAL TEST MATERIALS** - During the final comment period, the importance of using materials of high quality that are standardized and analytically defined was emphasized. In CHD, the need for free fatty acids for intravenous preparations and the need

for studies of longer duration with smaller doses was voiced. It was generally felt that dose-response studies are needed and valid controls are critical. In terms of controls, consideration should be given to studies having three treatments: omega-3 fatty acids, omega-6 fatty acids, a third group consisting of either a non-caloric placebo or something other than omega-6 fatty acids. An initiative aimed at supporting research needed in population directed outcomes such as diabetes with myocardial infarctions as a major risk group should be made. The products needed for research studies include those available through the BTM program and larger quantities of purified EPA and DHA for clinical studies, which may often require 5 kg quantities. In terms of evaluating the Biomedical Test Materials Program, it was concluded that the products currently produced are well-suited to current needs except for increased quantities of purified EPA and DHA must be produced. It was felt that the products should be available both with and without antioxidants and some studies should evaluate omega-3 fatty acids without vitamin E. It was also suggested that the availability of materials should be more widely publicized within the research community.

MEETING PARTICIPANTS

Committee Members:

1.	Dr. John Wallingford	Office of Special Nutrition, FDA
2.	Dr. William Connor	Oregon Health Sciences University
3.	Dr. Dwight Robinson	Massachusetts General Hospital
4.	Dr. Rashida Karmali	Memorial Sloan Kettering Cancer Center
5.	Dr. Patricia Fair	National Marine Fisheries Service, DOC
6.	Dr. Norman Salem, Jr.	NIAAA, NIH
7.	Ms. Beth Aurecchia	National Marine Fisheries Service, DOC
8.	Dr. Susan Pilch	NCI, NIH
9.	Dr. Momtaz Wassef	NHLBI, NIH

Other Federal Government:

11.	Dr. William Lands	NIAAA, NIH
11.	Dr. Paddy Wiesenfeld	Office of Special Nutrition, FDA
12.	Dr. Lawrence Lin	FDA
13.	Dr. Isabel Chen	Office of Pre-Market Approval, FDA
14.	Dr. Ronald Innerfield	Division of Metabolic & Endocrine Product, FDA
15.	Dr. Gloria Troendle	Division of Metabolic & Endocrine Product, FDA
16.	Dr. Orville Levander	Human Nutrition Research Center, USDA
17.	Dr. Norberta Schoene	Human Nutrition Research Center, USDA
18.	Dr. Melvin Mathias	CSRC, USDA
19.	Dr. Joseph Judd	Agricultural Research Service, USDA
20.	Dr. Eugene Zimmerman	NIAID, NIH

Outside Experts:

21.	Dr. Alexander Leaf	Department of Preventative Medicine & Clinical Epidemiology, Harvard Medical School
22.	Dr. Howard Knapp	Division of Clinical Pharmacology, University of Iowa
23.	Dr. Artemis Simopoulos	Center for Genetic, Nutrition and Health
24.	Dr. David Rose	Division of Nutrition and Endocrinology, American Health Foundation
25.	Dr. Lawrence Harker	Emory University, Division of Hematology & Oncology
26.	Dr. Richard Sperling	Brigham and Women's Hospital, Department of Rheumatology and Immunology
27.	Dr. John Bagdade	Department of Medicine, Rush Medical College
28.	Dr. Susan Carlson	University of Tennessee, Newborn Center

MEETING OBJECTIVES

A meeting of the Fish Oil Test Materials Committee (FOTMAC) was convened on May 12, 1994. Experts in the field of health effects of omega-3 fatty acids were invited to help assess recent progress. The goals for the May 12 meeting were to review the omega-3 research data with respect to health effects and to evaluate the Fish Oil Test Materials Program. Scientists in six key areas (heart disease, thrombosis, inflammation, diabetes and metabolism, neural development and cancer) were invited to present a 20 minute synopsis of recent progress in their field. A member of the FOTMAC together with outside experts then led a discussion of the topic involving all participants. The intent of the meeting was to define research needed to be conducted and the materials needed. A good representation of the various government agencies involved in the funding and regulation of lipid research attended. Representatives from each of the NIH Institutes were present as well as individuals within the Food and Drug "sides" of the Food and Drug Administration.

The format of the meeting and instructions to the presenters was as follows:

Synopsis by Presenter (20 minutes)
Please summarize in "abstract form" the relevant findings in your area. Present your own research findings if they relate to the overall argument. Indicate the validity of the research design, methods, results, and conclusions and the confidence that should be given to the results and conclusions. Where you find research designs to be inadequate, please indicate guidelines from more proper studies. Also, please give us your assessment of the major information gaps in your area. What are the impediments, if any, towards defining: a) definitive conclusions on the health effects of omega-3 fatty acids in this research area? b) clinical recommendations? Please summarize in what capacity the Biomedical Test Materials Program can further research in this area.

Discussant Critiques (10 minutes)
Please critique the research area, developing any points that were not adequately brought out by the presenter. You may begin with a statement of your own, if desired, and then you may question the presenter to help focus on important issues. You will then be asked together with any co-discussants to lead the general discussion. It is imperative that the discussants keep their remarks and the general discussion focused upon key issues so that it is possible to adhere to our schedule.

General Discussion (10 minutes)
There will be an opportunity for the expert group to comment and ask questions. There will not be time to discuss research issues in detail other than when they are pertinent to the overall interpretation of important experiments. It is hoped that by the end of the discussion, some consensus can be reached concerning the status of each sub-area to be considered.

MEETING AGENDA

I. Meeting Objectives (N. Salem)

II. Program Status (P. Fair)

III. Status Reports on Omega-3 Fatty Acid Related Federal Activities
- A. NIH Institute Grant Activity
- B. FDA Activities
- C. USDA Activities

IV. Synopsis of Recent Progress in Omega-3 Fatty Acid Research
- A. Heart Disease & Arrhythmia (A. Leaf)
  discussants: (M. Wassef , R. Innerfield)
- B. Thrombosis (L. Harker)
  discussants: (H. Knapp, N. Schoene)
- C. Cancer (D. Rose)
  discussants: (R. Karmali, S. Pilch)
- D. Intermediary Metabolism & Diabetes (J. Bagdade)
  discussants: (W. Connor)
- E. Inflammation/Cytokines (R. Sperling)
  discussants: (D. Robinson)
- F. Neural Development (S. Carlson)
  discussants: (J. Wallingford, W. Connor)

V. Discussion of Future Directions for Program (all)
- A. Materials Needed
- B. Research Needed
- C. Conclusions/Recommendations

BIOMEDICAL TEST MATERIALS PROGRAM

Patricia A. Fair, Ph.D

Overview

The Biomedical Test Material Program (BTM), is a cooperative program between the National Oceanic and Atmospheric Administration (NOAA), National Marine Fisheries Service (NMFS) and National Institutes of Health (NIH), National Institute of Alcohol, Drug Abuse, and Alcoholism (NIAAA). The BTM Program provides the technical development and production of research grade materials for the investigation of omega-3 fatty acids. The BTM Program, in operation since 1987, has supported over 220 research studies including 77 clinical projects using these materials. The majority of these are grantees from the National Institutes of Health. There have been studies performed on various animal species, *in vitro* studies, and human studies. Currently, 43 researchers hold an Investigative New Drug Number (IND) for conducting human studies using the test materials. In addition, there have been four human studies conducted in other countries. In addition, academic and industry requests for test materials not directly used in the biomedical field are supplied by the NOAA/NMFS Charleston Laboratory. Thus far, 67 researchers have received test materials under this area. Further, many analytical laboratories have received test materials for use in calibration and standardization of lipid analyses. These test materials are being utilized by U.S. and foreign investigators for research on cardiovascular diseases, arthritis, diabetes, inflammatory and autoimmune diseases, kidney disorders, cancer, malaria, skin disorders and lipid metabolism. This program has helped to generate high quality research in this area leading to a better understanding of the health effects of omega-3 fatty acids and helped to set standards for the commercial sector. The Charleston Laboratory, NOAA thus has made a considerable investment in facilities, personnel, training and time in developing this program to purify marine oils into various products. The Memorandum of Understanding (MOU) with NIH provides cooperation between a leading laboratory at the NIH involved in fatty acid research and encourages a two-way transfer of expertise in this area. The BTM Program provides support for an undervalued industry (menhaden) by the production of biomedical test materials from menhaden oil that will lead to a better understanding of the role of the unique marine fatty acids (omega-3's) in nutrition and health. The program's goal is to help attain a conclusive research decision on the effects on omega-3 fatty acids based on objective, evaluation of results and to further the design and conduct of studies to yield such results.

Establishment of the BTM Program

A conference in 1985 on "Health Effects of Polyunsaturated Fatty Acids in Seafoods" investigated the role of omega-3 fatty acids in modulation of human metabolism as well as their role in the prevention and treatment of a number of diseases. The conference had two objectives: (1) to review research data on the health effects of polyunsaturated fatty acids (PUFAs) in seafoods and the impact on eicosanoid formation, thrombosis, inflammation, and the role of docosahexaenoic acid (DHA) in membrane function; and (2) to develop a research agenda to determine the range of effects of PUFAs of seafood origin in the American diet. This conference concluded that additional basic information from biochemical and clinical studies was needed prior to formulating recommendations. A thorough examination of the problems associated with meeting research objectives led to the conclusion that the lack of a highly purified, quality-assured omega-3 PUFA compounds needed to conduct large-

scale studies, combined with their high cost on commercial markets were limiting factors in omega-3 research. Long-term, systematic studies using standardized, quality-controlled omega-3 test materials were lacking. This need was realized within NOAA and a program was formed to meet this challenge. The establishment of a MOU between, NOAA and NIH, prepared the framework for providing a long-term, consistent supply of test materials to facilitate the evaluation of the role of omega-3 fatty acids in health and disease. The agencies agreed to cooperate on, and provide support to, research activities related to the biological mechanisms by which a seafood diet or the ingestion of fish oils may influence health. The NOAA/NMFS Charleston Laboratory is committed under this MOU to provide a long-term, consistent supply of quality-assured test materials. Since the availability of high quality test materials was viewed as a critical element, NMFS embraced the opportunity to fill this need and to give direction to research agendas. A commitment was made in 1985 for NOAA/NMFS to provide research test materials of known composition, free from contaminants, and in the quantity required for studies over their duration. Proper evaluation of lipid components found in seafood and fish oils require that specific, quality-controlled test materials be made available to researchers. A strategic plan was formulated utilizing internationally recognized academic, clinical and research managers to support research agendas relative to the biochemical action of omega-3 lipids. The NIH serves in an advisory capacity on research and technical issues.

Strategic elements of the BTM Program consisted of a controlled production facility, extensive quality assurance, packaging and distribution of omega-3 fatty acid test materials. The joint administration of the program was by a committee formed in recognition of the MOU. The committee was structured to provide advice from a broad base of research scientist and managers from academic and government institutions, including individuals of international reputation and knowledge in the chemistry, biochemistry, and nutrition of omega-3 lipids, particularly in the conduct of human nutrition and disease. A distinguished board of internationally recognized academic, clinical, and research managers formed the Fish Oil Test Material Advisory Committee (FOTMAC). The FOTMAC is chaired currently by Dr. Salem (NIAAA) and is composed of scientists representing the funding agencies (NIH), the research community, Department of Commerce (DOC), and the Food and Drug Administration (FDA). The FOTMAC provides scientific advice to the DOC regarding the types of materials needed by research scientists, shipping procedures for the materials, and additional quality control and production issues. In addition, a Fish Oil Test Materials Distribution Committee (FOTMDC) was formed to process the applications received from investigators. The FOTMDC, composed of NIH and other Federal scientists, advises the DOC of applicants who have fulfilled the application process, and makes recommendations regarding the distribution of requested materials. Applications are considered from researchers having funded, peer-reviewed research studies indicating a need for the requested materials.

The construction of the NMFS pilot production facility began in 1986 and was operational in September 1987. Concurrently, an extensive quality assurance program, methods for handling, storage, and distribution, and packaging of these labile products was developed. In 1988, production of omega-3 ethyl ester concentrate was begun. The NMFS Charleston Laboratory was inspected by FDA and certified as an approved manufacturer of fish oils. In addition, during 1989 extensive support information for human studies, in the form of a Drug Master File (DMF), was prepared and

filed with FDA. The DMF assists researchers applying for an investigation new drug (IND) number to conduct research studies. In 1990, production of purified EPA and DHA allowed the use of these highly valuable materials in research studies. The test materials are distributed on a gratis basis to researchers upon the recommendation of the FOTMDC. With the availability of consistent quality fish oil test materials, researchers are able to reach a new level of confidence in their data. The availability of materials for the duration of the study allow the design and conduct of long-term projects. The NMFS provides technical information on diet preparation, methodology, product stability, handling, and storage. It is anticipated that these actions help to set the pace for the production and quality assurance of caliber fish oils in the commercial sector.

Program Scope

The 1986 MOU between NIH/NIAAA and NOAA/NMFS was supported by a congressional increase of one million dollars to the NOAA budget in FY1986. The commitment was for a multi-year production of omega-3 test materials to serve as research material for biochemical, animal, and clinical models. Quality-assured test materials verified to be free of contaminants and other interfering materials are provided over the multi-year course of investigations. In successive years the Program has received $937K/yr (one million minus the Gramm-Rudman-Hollings cut). During the first two years, major equipment purchases were made to complete the pilot plant installation. Thereafter, the major budget items consisted of personnel and supplies to operate the Program. Costs each year are allocated for ton quantities of raw materials, maintenance and repairs of equipment and instrument, quality-assurance and distribution. Funding has decreased over the years with $814,000 in the FY93 budget. The facilities of the BTM pilot plant encompasses approximately 2590 sq. ft including storage facilities, laboratories, and freezer space. The BTM facility has a 2-stage wiped film molecular still, glass/teflon reaction vessels, crystallizer, evaporation system, separatory funnels, supercritical CO_2 unit, and a preparative High Performance Liquid Chromotography systems. In addition to the BTM pilot facility, there are several support laboratories devoted to lipid analyses, polychorinated biphenyls/pesticides, metals, and general chemical analyses. Quantities of test materials are supplied on a gratis basis in the following amounts: 500 kg vacuum-deodorized fish oil, 50 kg n-3 concentrate, 175 g eicosahexaenoic acid (EPA) and 100 g DHA. Studies requiring large volumes of test materials for research are requested to include costs in their project's budget.

Production

Key elements in production are a consistent supply of single-source raw material (menhaden oil) for processing, optimized operating parameters, use of a nitrogen environment during processing, and handling and storage techniques designed to protect materials.

Quality Assurance

Each lot of material requires 29 analyses that provides assurance in standard chemical composition, absence of contaminants and artifacts, and minimal oxidation. Specifications are set for acceptance of both raw materials and the finished product. Analyses are provided for placebo materials procured by researchers from other sources to assure that antioxidant levels are balanced with BTMs obtained from the program. In addition, long-term stability testing is also performed.

Distribution

Test materials are available for the duration of the research project. This allows researchers to design and execute long-term projects with confidence in their supply. The materials are provided in the quantity required with packaging and dosage delivery customized according to need. Bulk-packaging is designed for optimal use and storage of the material. Soft-gel capsules are matched with placebos for double-blind clinical studies. Preparation of the Drug Master File was instrumental in expediting the approval of the use of omega-3 fatty acids in human clinical studies. Manuals on Analytical Methods for the Quality Assurance of Fish Oil, Production Methods/Safety and Distribution were produced by the DOC and are available to investigators.

Technical Information

A number of technical services and information are afforded to researchers receiving test materials. This includes information on experimental diet preparation, methods for balancing antioxidants, product delivery form, product stability, recommended storage and handling procedures, analytical procedures, use of placebos in double-blind studies, export requirements for drug, and assistance in obtaining IND approvals.

Test Materials Available

- **Vacuum-deodorized menhaden oil in bulk and soft-gel capsules**
- **n-3 ethyl ester concentrate, prepared from winterized Atlantic Menhaden (*Brevoortia tyrannus*) oil, in either bulk or soft-gel capsules**
- **Encapsulated commercial preparations of corn, olive, and safflower oils**
- **Encapsulated and bulk preparations of ethyl esters of corn, olive, and safflower oils**
- **Encapsulated and bulk preparations of purified EPA and DHA ethyl esters (>95% purity) prepared from menhaden oil.**

STATUS REPORTS ON OMEGA-3 FATTY ACID RESEARCH

Committee Reports:

- **National Cancer Institute (NCI)** - It was reported that NCI is funding epidemiological studies, of which only one is a human study. The fifteen grants currently funded represent a decrease from past levels.

- **National Heart, Lung and Blood Institute (NHLBI)** - The funding for omega-3 fatty acid research began in 1983 with $300K in funding. This increased in 1984 to $566K and to $1M in 1985. In 1986, a Request For Applications (RFA) was announced and the number of grants peaked with $2M of research funding. Research funding attained $4M in 1990 and has declined to $750K for 1993 and 1994. Currently, 5 grants are funded that relate to omega-3 fatty acids. Approximately, 15-20 omega-3 grants have been seen within a five year period. A peak of fish oil applications occurred in 1989 with 16 grants. Dr. Momtaz commented that we need to move omega-3 research into the 21st century of molecular biology. Replies to this comment focused on the fact that omega-3 research is a rather young field and we need to know more about the physiology first.

- **National Institute of Allergy and Infectious Disease (NIAID)** - Dr. Zimmerman said that the interest in fish oils relates to its potential to suppress inflammation from allergy and infectious disease via the membrane altering properties of omega-3 fatty acids. This agency is funding three grants directly on omega-3 research and another three peripherally related to omega-3 research. NIAID funding was approximately $1.4M for this research.

- **National Institute of Alcohol Abuse and Alcoholism** - The NIAAA has one grant that directly relates to omega-3 research.

- **Food, and Drug Administration** - Dr. Innerfield reported that overall funding in this area decreased and shifted to molecular biology. He suggested that researchers focus on metabolic biology. However, committee members agree that more needs to be discovered about the physiological effects and clinical benefits of fish oil to determine the area of metabolic biology to pinpoint. Dr. Innerfield addressed design questions concerning omega-3 research studies. One problem is that studies have been small and lack statistical robustness. Other difficulties seen in omega-3 research studies is whether the placebo serves as an adequate comparison. He suggested that calories should be considered and suggested the possibility of targeting a placebo without calories, such as olestra. Dr. Lin presented an update on the fish oil petition submitted for GRAS (generally regarded as safe) approval in 1986. The maximum amount allowed in food was 30%, but was later reduced to 20%. An agreement for the maximum use level set in food products is for 3 g EPA and DHA/day, and currently the petition is under general council review at the FDA. It is not known when it would be approved. Dr. Wallingford spoke on food labels concerning omega-3 fatty acids. The boxed label has tight controls over what information can be presented and can only say how much n-3 fatty acids are present. It is possible that information appearing outside of the nutrition

label could contain substantiated health claims which then could be directed toward providing benefits for a subpopulation. However, a health claim relating to the effect of omega-3 fatty acids to heart disease cannot be made since the research is inadequate. He noted three research areas that were lacking in making health claims for cardiovascular disease as: 1) questionable usefulness of intermediate markers; 2) studies directed at subpopulations that may be beneficial and, 3) accepted markers were questioned.

- **United States Department of Agriculture** - The USDA funded 17 projects on fatty acid research at $10M in 1994. There were four human studies. Five studies were specifically directed at omega-3 fatty acids funded at $3M.

AGRICULTURAL RESEARCH SERVICE (ARS) INTRAMURAL	
FY94 17 PROJECTS (Fatty Acid Research)	10.5M
OMEGA-3 COMPONENT (50%)	5.3M
COOPERATIVE STATE RESEARCH SERVICE (CSRS) EXTRAMURAL	
FY92 FEDERAL FUNDING (Fatty Acid/Cholesterol)	2.5M
FY92 NON-FEDERAL FUNDING (State/Grants)	8.9M
SUBTOTAL	11.4M
OMEGA-3 COMPONENT (20%)	2.7M
ESTIMATED TOTAL EFFORTS	8.0M
(CORRECTED, NON-FEDERAL)	7.0M
HUMAN NUTRITION INFORMATION SERVICE, HNIS	
NUTRIENT DATA BASE	
OMEGA-3 FATTY ACID COMPOSITION OF FOODS	

Summary

While there is substantial amount of funding by the federal government for omega-3 research, it is obvious that there is a decrease in the number of fish oil studies submitted for government funding as well as a decrease in overall research spending. These decreases are likely due to a number of factors such as a lack of NIH Request For Application (RFA) seeking to stimulate investigator-initiated research in the omega-3 research area as well as a lack of research funds within funding agencies.

SYNOPSIS: Effects of Omega-3 Fatty Acids On Atherosclerosis and Arrhythmias

Alexander Leaf, M.D.

Since the initial reports by Bang and Dyerberg on the possible role of omega-3 fatty acids in the low incidence of coronary heart disease (CHD) mortality among the Greenland Inuits (1,2), most attention has been directed to the possible role of these long chain, polyunsaturated fatty acids on atherogenesis. Only recently, and in many fewer studies, effects of these fatty acids on cardiac arrhythmias have appeared.

I. Possible Antiatherogenic Effects:

A. Effects on Plasma Lipids

There have been many reports on the effects of fish oils on blood lipid profiles. There is very little effect on total or low density lipoprotein (LDL) cholesterol levels unless the prior diet had been high in saturated fats and the fish oil substituted for the saturated fatty acids, in which case the total and LDL cholesterol levels may drop markedly, as is the case with the omega- 6 PUFA. High density lipoprotein (HDL) cholesterol is generally unchanged. There is an invariable large reduction in serum triglycerides that is dose dependent. This is associated with a reduced very low density lipoproteins (VLDL). Both result largely from a reduction in hepatic synthesis of triglycerides and apoliprotein B. There are changes in the physical characteristics of the lipoproteins, such as in their melting points and size, but the effects of these changes on the atherogenicity of these lipids in humans is still undetermined.

B. Effects on Factors Affecting Atherogenesis

In both *in vitro* and *in vivo* studies a number of potent factors involved in atherogenesis are modulated by the ingestion of fish or by supplements of omega-3 fatty acids. Interestingly most of these are proatherogenic factors which are suppressed or their actions are diminished by omega- 3 PUFA, but some are antiatherogenic and their effects are accentuated by omega-3 fatty acids. Some of these factors, their response to omega-3 PUFA and the functions which they modulate are summarized in Table 1.

Table 1

FACTORS AFFECTING ATHEROGENESIS

Factors **Function**	**Omega-3 fatty acid:effect of**
1. Thromboxane, TXA_2,(2)	platelet aggregation (2) vasoconstriction
2. Prostacyclin, PGI, (3)	prevents platelet aggregation vasodilation
3. Leukotriene, LTB_4,(4)	neutrophil chemoattractant and aggregator
4. Tissue Factor (5)	blood clotting factor
5. Platelet activating factor, PAF, (6)	activates platelets
6. Platelet derived growth factor, PDGF,(7)	chemoattractant and mitogen for smooth muscles and macrophages
7. Superoxide formed by leukocytes (8)	cellular damage, enhances LDL uptake by macrophages
8. Interleukin-l, IL-1, tumor necrosis factor TNF (9)	expresses endothelial Adhesion Molecule-1, stimulates PAF, inhibits plasminogen activator, stimulates smooth muscle cell proliferation, stimulates neutrophil superoxide formation
9. Endothelial derived relaxation factor, EDRF, (10,11)	reduces arterial constrictor responses protects endothelial surface from thrombi

In addition to these factors important in atherogenesis, a number of physiological, and perhaps pharmacologic, actions of these omega-3 fatty acids have been demonstrated. Some are summarized in Table 2.

Table 2

ADDITIONAL PHYSIOLOGIC AND PHARMACOLOGIC EFFECTS OF FISH OIL.

1. Decreases blood pressure in normal and moderately hypertensive subjects (12).
2. Decreases blood viscosity (13).
3. Decreases microvascular albumin leakage in insulin dependent diabetics (14).
4. Decreases plasma triglycerides (15).
5. Decreases vascular response to norepinephrine (16).
6. Decreases platelet adhesion (17).
7. Decreases leucocyte/endothelial interactions (18).
8. Increases vascular compliance (19).
9. Increases thrombolytic activity of TPA (20).
10. Increases platelet survival *in vivo* (21).

Again one notes the interesting combination of a decrease in effects which are regarded as atherogenic and enhancement of antiatherogenic actions by these omega PUFA. This would seem to indicate a probable common denominator effect on cellular physiology, biochemistry, or structure which is important to many cellular functions. This in turn suggests a long-term evolutionary role of these PUFA, as we recognize must have been the case with their omega-6 counterpart, arachidonic acid and its eicosanoids.

There have been a great many animal studies in which the effects of the omega-3 PUFA have been examined in many species in very many different protocols on experimentally induced atherosclerosis. Space doesn't permit review of individual studies. It is clear that if one studies animals lacking LDL receptor function, as the Watanabe rabbit, no protection is achieved with omega-3 fatty acids. In others reduction of the atheromatous changes in the vascular wall has been demonstrated (22-30). A recent study (5) on baboons with dietary hypercholesterolemia found a marked protective effect against intimal proliferation on the site of endarterectomy.

Despite the biochemical and physiologic effects of the omega-3 fatty acids, there is sparse evidence in adequate prospective, placebo controlled, double blind clinical trials of their effects on atherosclerosis or health in humans. The epidemiologic studies on the Greenland Eskimos (31) have received corroboration from a Japanese study (32) examining the mortality from CHD in a fishing village as compared with that in an inland farming village. Importantly, in this study the incidence of cerebral vascular mortality as well as that of CHD was lower in the fishing than in the farming village. This finding, in a country with a high prevalence of cerebrovascular disease, where the disease is well recognized and diagnosed, reassures us that a high intake of fish oils does not result in an increase in cerebrovascular accidents.

There have been several retrospective dietary studies reporting the effects of dietary fish intake on mortality from CHD. The Zutphen trial (33) stimulated these investigations and showed that the ingestion of small amounts of fish daily was associated with a graded marked reduction of relative risk for mortality from CHD over a period of some 20 years in 852 middle-aged men who

were free of CHD on entry. Other similar studies confirmed this finding but some failed to see the benefits (34-37). Now that it has been shown that a simultaneous high intake of omega-6 and of saturated fat can negate potential health benefits of omega-3 fatty acids, there is a possible explanation for the contradictory reports. The most careful of these retrospective dietary reviews, however, was the subgroup analysis of the fatty acid intake in the 6255 men in the Usual Care control group for the MRFIT study (38), in which Dolecek and Grandits, showed an inverse correlation between the dietary intake of the omega-3 PUFA and all cause and CHD mortality. With four dietary reviews during the seven year course of this study, the quality of the data is high.

The other evidence from human studies, with one exception, comes from the ten clinical trials (39-48) in which the effect of fish oil supplements has been studied on the incidence of restenosis following percutaneous transluminal coronary artery angioplasty (PTCA). Although the combined first seven reports resulted in a meta-analysis indicating marginal benefit, this has been negated by the following three reports which have all been negative. Still questions remain regarding the adequacy of the protocols used in these studies. It is important to realize that adequate time is required to achieve incorporation of the omega-3 PUFA in phospholipids of membranes in cells, which turn over slowly, prior to the PTCA procedure and the necessity to curtail both saturated and omega 6 fatty acid intake to maximize the opportunity for potential beneficial effects to occur (48).

A possible effect of fatty acids on cardiac arrhythmias has been studied for several decades. Definitive evidence has come from the careful studies of Charnock and McLennan (49) in Australia. They have shown that occurrence of fatal ventricular arrhythmias in rats with experimental myocardial infarctions can be largely prevented by diets containing omega-3 fatty acids. In marmosets, they showed a similar protective effect on the threshold for induction of arrhythmias on direct electrical stimulation of the hearts (50). Stimulated by these studies, my laboratory has shown that both of the long chain omega-3 PUFA, EPA, and DHA modulate the L-type calcium channels in the sarcolemma of isolated heart cells and this action correlates with prevention of toxic arrhythmias induced by ouabain toxicity (51,52). These results obtained *in vitro* on cultured neonatal rat cardiac myocytes have now been confirmed by patch-clamp direct measurements of the calcium currents through L-type calcium channels and on contractility of adult rat myocytes (53). In the course of these studies, we learned that the free fatty acids added to the media of the isolated myocytes exert their anti-arrhythmic effects so promptly as to exclude the likelihood that incorporation in to membrane phospholipids is essential for their protective effects. The protective effect is not restricted to arrhythmias caused by toxic agents, as it has now been shown that infusion of an emulsion containing largely the two long chain omega PUFA, EPA and DHA, just prior to inducing ischemia in a prepared dog model will prevent the occurrence of ischemia induced fatal ventricular fibrillation (54).

In a carefully designed DART (Diet and Reinfarction Trial) study, Burr and associates (55) showed that ingesting fatty fish two or three times weekly by male subjects following a myocardial infarction was associated with a reduction in total mortality from heart attacks but not in the incidence of cardiac events, angina or infarction. These results suggest that the beneficial effect of omega-3 fatty acids in the DART study prevented arrhythmias ensuing during acute myocardial ischemia - the major cause of death from heart attacks in the United States and Western industrialized countries. This gives us hope that what has been learned in the animal studies will have beneficial effects as well

for humans. Appropriate clinical trials in humans are now urgently needed.

III. Conclusions:

1. There is much encouraging evidence to indicate that dietary intake of omega-3 fatty acids may prevent human atherosclerotic diseases of which coronary heart disease (CHD) is its most prevalent clinical expression.

2. There is increasing evidence that fatal cardiac arrhythmias may be prevented by dietary or intravenous administration of the omega-3 fatty acids, EPA and DHA.

IV. Recommendations:

1. Human prospective, randomized, placebo-controlled, double blind clinical trials are needed to test the efficacy of omega-3 fatty acids in preventing heart attacks, peripheral vascular disease, cerebrovascular disease, the clinical consequences of atherosclerosis. The duration and costs of such clinical trials could be considerably reduced by secondary preventive interventions, i.e. in patients with documented atherosclerotic disease. Furthermore, end points should include progression or regression of vascular atheromatous changes determined by quantitative methods such as angiography, ultra-sound, MRI, PET scan, etc., as well as by morbidity and mortality. Investigators should be aware that the effectiveness of ingested omega-3 fatty acids may be diminished unless total fats, saturated fats and omega-6 fatty acids are decreased in the diet. This should apply to both the experimental and the control cohorts. To demonstrate that any beneficial effect is not due simply to increased total polyunsaturated fatty acids ingested by the experimental group, the control should be given a supplement of either corn oil or safflower oil, but not olive oil.

2. Human studies are urgently needed to see whether arrhythmic deaths which annually account for some 300,000 of the 500,000 deaths from heart attacks in the U.S.A. alone, can be prevented both by feeding experiments and by infusion of emulsions of omega-3 fatty acids into patients at very high risk of impending fatal ventricular fibrillation. It is my opinion that this route provides the quickest test of the efficacy of the omega-3 fatty acids on a very important human disease.

3 To accomplish these goals, it is highly important that the source of highly purified and standardized preparations of fish oils and of the purified fatty acids, EPA and DHA, in mixtures and individually be sustained. The Biomedical Test Materials Program of the NIH and the Department of Commerce is essential for continuation of both the clinical trials and the necessary research on the basic mechanisms responsible for the important biologic effects of these PUFA. As the preparations have become more highly concentrated in the longest and most unsaturated of the omega-3 fatty acids, identification of EPA and DHA as the active ingredients of fish oils has become possible. Because EPA and DHA may differ in their potency, or even possess opposing biological effects, it will be increasingly important that the two fatty acids be prepared individually in amounts that will allow clinical and laboratory testing of their comparative effectiveness.

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DISCUSSION COMMENTS: Effects of Omega-3 Fatty Acids on Atherosclerosis and Arrhythmias

Momtaz Wassef, Ph.D.

Atherosclerosis underlies most coronary heart disease (CHD), a major cause of death and disability, as well as much peripheral vascular disease, many cases of stroke, and several other diseases. Atherosclerosis is a multifactorial, polygenic process where complex interactions between dietary factors and genetics play a prominent role in the development of the disease.

There is evidence to suggest that dietary omega-3 fatty acids might be important in the "prevention" of CHD. Many fundamental questions remain unanswered which cast serious doubts about the therapeutic or health effects of these fatty acids. For example, from the nutritional point of view, do the epidemiologic studies reflect a reduction in saturated fatty acid intake, an increase in omega-3 fatty acid intake, or both? How much of which fatty acid should be given for a specific purpose? For how long? How does the fat content of the basic diet influence the response to omega-3s? As a matter of fact, we still lack controlled evidence of functional benefit from omega-3 fatty acid supplementation in any human disease other than the rare cases of apparent omega-3 fatty acid deficiency (see Fitzgerald, Knapp and others).

Owing to the limited time and space, it will do no justice to summarize the status of the basic science or the clinical investigations in the field. However, it must be pointed out that only very few effects of omega-3 fatty acids (such as lowering plasma triglyceride levels) are consistently reproducible by all investigators. Almost all other basic science and clinical investigations report predominantly observational or descriptive data that are often inconsistent or debatable. This is likely due to the lack of systematic investigations, and the difficulty in obtaining consistently high quality omega-3 fatty acids.

Now that the difficulty in obtaining high quality fatty acids has been solved, the systematic investigation of the effects of omega-3 fatty acids should be well planned. Recent studies indicate that there are some known genes (and others as yet uncharacterized) that are responsive to diet and play an important role in atherosclerosis. Advances in cellular and molecular biology and genetics and the capability for creating genetically manipulated animal models offer an opportunity for in-depth investigation of the complex diet/genetics interactions in the presence and absence of one or more risk factors, and opportunities for discovering new genes, loci and traits that may play an important role in atherogenesis. I believe that the field needs state-of-the-art research at the molecular level **combined with sophisticated** pathophysiological investigations. Attempts to design or plan clinical trial(s) without an adequate science base would again prove fruitless. Accordingly, pure and highly purified omega-3 fatty acids should be made available for basic science studies, and may be for **small** clinical investigations. I also think that the technologies for manufacturing these fatty acids have been well developed and reliable enough to entertain the idea of transferring this task from the federal government to the private sector.

SYNOPSIS: Antithrombotic and Antiproliferative Effects of n-3 Fatty Acids

Laurence A. Harker, M.D.

Effects of Eicosanoids and Platelets

Substantial information is now available on the effects of omega-3 fatty acids on a variety of cellular processes involved in vascular reactivity, atherogenesis, and thrombosis (1-4). Extensive investigations show that dietary omega-3 fatty acids prolong the bleeding time and suppress platelet aggregation *ex vivo* in both normal subjects and patients with hyperlipidemia (5). Platelet aggregation to low dose collagen is most frequently reduced and is associated with decreased platelet production of thromboxane A_2 (TxA_2) in a non-dose dependent fashion. Recently, dietary fish oil has been reported to inhibit platelet adhesion to collagen or fibrinogen-coated artificial surfaces (6,7). Although platelet adhesion to everted segments of rabbit aorta was not inhibited by feeding fish oil to human subjects, the bleeding time was prolonged (8).

Prostacyclin (PGI_2), produced by human blood vessels and cardiac tissues, is significantly increased in patients receiving fish oil prior to undergoing coronary artery bypass grafting (9). In these patients, despite inhibition of *ex vivo* platelet aggregation, serum TxB_2, and bleeding times, excessive bleeding was not observed during or after surgery.

Platelet-vascular interactions may also be inhibited in patients with vascular disease when given n-3 fatty acids in their diets. Radiolabeled platelet survival is reported to be prolonged in atherosclerotic patients with both normal and elevated plasma lipids (10,11). Increased urinary excretion of PGI_2 and thromboxane metabolites may also reflect heightened platelet-vascular interactions. Elevated levels of these prostaglandin metabolites in men with atherosclerosis were suppressed by large doses of dietary fish oil (for example, 10 g of omega-3 fatty acids per day) (12). Recently, the increase in urinary prostaglandin metabolites following percutaneous transluminal coronary angioplasty (PTCA) has been reported to be suppressed by dietary fish oil (13).

Effects on Cellular Function

Dietary fish oil or omega-3 fatty acids may have important effects on several cellular systems potentially important in atherogenesis. Macrophages produce less interleukin-1, tumor necrosis factor, and tissue factor after loading with omega-3 fatty acids; and endothelial cells produce reduced amounts of fibroblast growth factor and PDGF-like material (14-16). G-CSF and GM-CSF synthesis are also decreased in stimulated endothelial cells loaded with EPA as compared to oleic acid (17). Dietary fish oil reduces leukocyte-endothelial interactions following the systemic administration of oxidatively modified low density lipoprotein (18). Monocyte adhesion and migration are reduced by omega-3 fatty acids, and these cells have a well documented role in early plaque formation. Omega-3 fatty acids reduce monocyte and neutrophil production of toxic oxygen metabolites (free radicals) (19-23), and attenuate free radical generation in a rabbit model of coronary occlusion-reperfusion (24).

Endothelial cells are another important cellular element modulating atherogenesis. Endothelial cell production of platelet derived growth factor, a powerful mitogen and inducer of smooth muscle cell migration, is inhibited by exposure to omega-3 fatty acids (16). All of these cellular effects of omega-3 fatty acids may contribute to inhibiting atherogenesis at a cellular level. Endothelium-derived relaxing factor activity is enhanced by dietary omega-3 fatty acid supplementation, and this activity has been observed to be impaired in atherosclerotic arteries (25). NO-mediated enhancement of arterial relaxation may help reduce endothelium-dependent vasospasm. NO-dependent inhibition of platelet responsiveness may represent another potential benefit of omega-3 fatty acids in vascular disease.

High dietary intake of omega-3 fatty acids may protect smokers from chronic obstructive pulmonary disease (bronchitis and emphysema) (26). Omega-3 fatty acids may be acting by interfering with neutrophil activation/ accumulation in the lungs of smokers. Such a mechanism may be related to a combination of reduced chemotaxis, lower production of leukotriene B_4, and decreased production of reactive oxygen intermediates. The actions of omega-3 fatty acids on activation of leukocyte platelets. These omega-3 fatty acids function to inhibit or slow down the interactions among platelets, neutrophils and monocytes, and vascular endothelium. Thus, these interactions may be characterized as antithrombotic and antiatherogenic via undefined mechanisms. Some of the actions of omega-3 fatty acids may be independent of eicosanoid production by affecting signal transduction networks distal to the actions of these lipid mediators.

Platelets from patients with severe arteriosclerosis may have defects in the mechanisms that control the amount of intracellular ionized calcium resulting in significantly higher resting levels of this signal (27). Platelet calcium homeostasis is abnormal in patients with severe arteriosclerosis, and platelets from rats fed fish oil have a lower basal flux of ionized calcium across the plasma membrane compared to platelets from rats fed corn oil (28). In addition to effects on calcium homeostasis in platelets, there are reports that demonstrate a protective effect of omega-3 fatty acids on cardiac glycoside induced toxic levels of intracellular ionized calcium (29). Perhaps these observations are related to the capacity of dietary fish oils to prevent ventricular fibrillation during occlusion-reperfusion (30). These mechanisms may help to explain the results of the secondary trial that demonstrated reduced mortality after consuming several fish meals per week (31).

Effects on Vascular Thrombogenicity and Vascular Lesion Formation

Dietary substitution and supplementation experiments with omega-3 fatty acids have been performed in rat, rabbit, quail, swine, and primate atherosclerosis models with conflicting results. In animal species where atherosclerotic plaques more closely resemble human lesions, such as swine and nonhuman primates, results have been more consistently favorable. Dietary omega-3 fatty acid supplementation reduced the extent of coronary atherosclerosis when given to swine in primary prevention studies (32,33). Several groups have evaluated omega-3 fatty acids in nonhuman primate models. When dietary omega-3 fatty acids are substituted for coconut oil in rhesus monkeys, a significant reduction in the extent of atherosclerotic lesion formation was seen at 12 months in the omega-3 fatty acid groups (34). Isocaloric substitution of fish oil for lard in African green monkeys also produced less coronary artery lesion formation in the fish oil group at 2.5 to 3 years (35). In a

secondary prevention study, fish oil or vegetable oil was added to an atherogenic diet or therapeutic (regression) diet given on an average of two years after commencement of an atherogenic diet in African green monkeys (36). The omega-3 fatty acid supplementation did not produce regression, and the vegetable oil group fared significantly better than the fish oil group on the regression diet. It appears that omega-3 fatty acids reduce atherosclerotic plaque development in swine and nonhuman primates when employed as a primary prevention, but their effect on secondary prevention or regression of established lesions needs further investigation. Dietary omega-3 fatty acid supplementation reduced intimal hyperplasia of peripheral or coronary autologous vein grafts in hypercholesterolemic dogs (37,38).

Because of discrepant claims regarding the relative biologic effects of omega-3 fatty acids, the effects of dietary omega-3 fatty acid on blood and vascular lipid composition, hemostatic function, blood thrombotic responses, vascular thrombus formation, and vascular lesion formation (VLF) have been measured in baboons (**39**).

Dietary omega-3 fatty acids displaced omega-6 fatty acids in plasma, platelets, blood vessels, and corresponding urinary eicosanoid metabolites ($p < 0.01$ in all cases) within weeks after initiating a semipurified diet containing 1 gm/kg per day n-3 fatty acid-ethyl ester concentrate (composed of two-thirds eicosapentaenoic acid, EPA, and one-third docosahexaenoic acid, DHA). Coincidentally, platelet hemostatic function became minimally impaired (template bleeding times prolonged from 4.3 ± 0.5 min to 7.6 ± 1.3 min, $p=0.039$; concentrations of collagen producing half-maximal platelet aggregation increased from 6.4 ± 2.1 to 8.5 ± 2.5 g/mL, p=0 045), and tissue factor expression by endotoxin-stimulated blood monocytes fell (from 6.5 ± 1.2 to 1.7 ± 0.14 mU/10^6 cells; $p<0.005$). Dietary omega-3 fatty acids decreased deposition of platelets onto thrombogenic segments of Dacron vascular graft incorporated into chronic exteriorized femoral arteriovenous (AV) shunts, a thrombotic process resistant to the effects of both aspirin and heparin (^{111}In-platelet deposition decreased from $14.1 \pm 1.4 \times 10^9$ platelets/5-cm and occlusion by 40 to 60 min, to $7.5 \pm 0.8 \times 10^9$ platelets/5-cm without occlusion; $p<0.001$). Platelet deposition onto segments of endarterectomized homologous normal aorta in the AV shunts of omega-3 fatty acid-treated animals was similarly reduced (from 4.4 ± 0.9 to $1.8 \pm 0.4 \times 10^9$ platelets; $p < 0.01$).

Dietary omega-3 fatty acids interrupted vascular thrombus formation at sites of surgical carotid endarterectomy (platelet deposition 1.5 ± 0.4 vs $4.4 \pm 1.0 \times 10^9$ platelets in untreated controls; $p<0.001$; Figure 1). Moreover, endarterectomized aortic segments from omega-3 fatty acid-treated donors exhibited little capacity to induce thrombus formation when tested in the AV shunts of control recipient animals (0.24 ± 0.10 vs $4.4 \pm 0.90 \times 10^9$ platelets). However, in the converse cross-over experiments, endarterectomized aortic segments from control animals actively accumulated platelets when studied in the AV shunts of omega-3 fatty acid-treated animals ($1.8 \pm 0.4 \times 10^9$ platelets; $p< 0.01$ vs omega-3 fatty acid-treated endarterectomized aortic segments in shunts of normal animals; Figure 1)

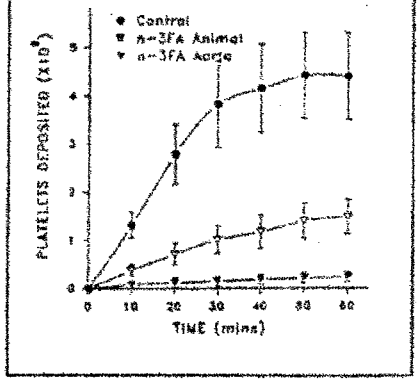

Figure 1. Graph showing impaired thrombogenicity of damaged arterial wall by dietary n-3 fatty acids (n-3FA) in baboons.

Dietary omega-3 fatty acids also abolished vascular lesion formation at sites of carotid endarterectomy 6 weeks postoperatively (cross-sectional area of neointima 0.048 ± 0.031 mm^2 compared with 0.428 ± 0.104 mm^2 in controls; p=0.010; Figure 2).

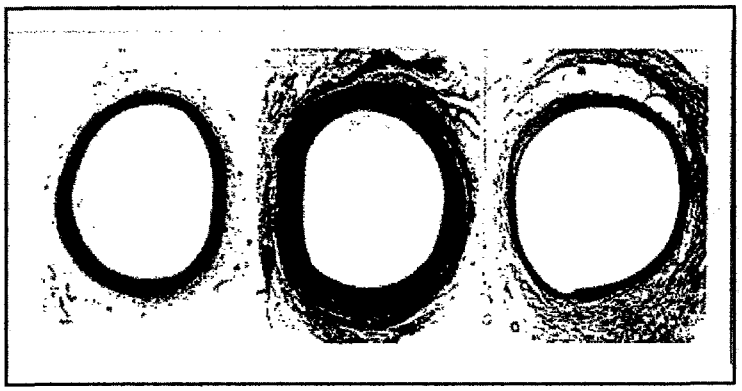

Figure 2. Photomicrographs showing effect of dietary omega-3 fatty acids on carotid endarterectomy lesion formation in baboons.

Thus, in nonhuman primates dietary omega-3 fatty acids in high dosage eliminate vascular thrombus formation and vascular lesion formation after mechanical vascular injury, while largely sparing hemostatic function and modestly reducing blood thrombotic responses. These effects are attributed to selective omega-3 fatty acid-dependent alterations in cellular membrane functions.

Thrombosis and Effects on Restenosis Following Angioplasty

Following percutaneous transluminal coronary artery angioplasty (PTCA), vessel occlusion may occur acutely as a result of intimal flap formation, subsequently as a result of thrombosis, or over one to six months as a result of a neointimal proliferative process. Smooth muscle cells migrate from the media to the intima, presumably in response to mitogens derived from platelets, endothelial cells, and activated macrophages. Accordingly, there has been considerable interest in the evaluation of omega-3 fatty acids in the prevention of PTCA restenosis. Important considerations in the evaluation of clinical trials of omega-3 fatty acids in PTCA include the dose of omega-3 fatty acids (relatively large doses are required to alter arachidonate metabolism), the timing of the initial dose in relation to PTCA (several days are required for full incorporation of omega-3 fatty acids into platelet and other cell membranes), blindness (the potential for bias in follow-up and designation of outcomes), an angiographic outcome (required for objective evaluation), must be obtained in a high percentage of subjects, angiogram reading must use quantitative technology and be independent of clinical information), and finally, sample size must be sufficiently large to detect clinically important improvement in the incidence of restenosis. Published clinical trials of omega-3 fatty acids have been carried out to assess the effects on restenosis following coronary angioplasty in Table 1. Although these data are as yet inconclusive, no substantial benefit is evident. Since angioplasty restenosis involves different processes than atherogenesis, the lack of convincing evidence for benefit of omega-3 fatty acids after angioplasty does not address the question of what anti-atherogenic benefits omega-3 fatty acids may produce in man.

Table 1.

Clinical Trials of Omega-3 Fatty Acids to Prevent Restenosis Following Coronary Angioplasty				
			Patients Assessed for Restenosis	
	Patients	omega-3 Fatty Acid, g/d	Fish Oil vs Corn Oil	Significance
Slack (40)	62	2.5	35 vs 39	NS
Dehmer (41)	82	5.4	19 vs 46	p=0.026
Grigg (42)	108	3.0	34 vs 33	NS
Reis (43)	186	6.0	34 vs 23*	NS
Milner (44)	194	4.5	19 vs 36* 22 vs 35*	p<0.008 p<0.04
Nye (45)	69	4.0	11 vs 30**	p<0.05
Bairati (46)	119	4.5	31 vs 48	p=0.05
FORT***	466	8.0	52 vs 47	NS

* Intention to treat
** Restenosis by lesion
*** Fish Oil Restenosis Trial (47)

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DISCUSSION COMMENTS: Antithrombotic and Antiproliferative Effects of Omega-3 Fatty Acids

Norberta Schoene, Ph.D.

Dr. Harker has written a synopsis that covers the major highlights of research results pertinent to the antithrombotic and antiproliferative effects of omega-3 fatty acids. Additional information on the effects of omega-3 fatty acids on the production of toxic oxygen metabolites and on calcium signalling can be found in the references included with the comments below.

References 1-4 are included to supplement the discussion of monocyte production of toxic oxygen metabolites (See Effects of Cellular Function Section, p 19). In addition, the observation that monocytes/macrophage produce less reactive oxygen species after omega-3 supplementation has also been reported for neutrophils (5). These reactive oxygen species (when over-produced) are receiving renewed attention as mediators of disease processes. Also, inclusion of a reference for the reduced production of platelet aggregating factor (PAF) after omega-3 ingestion would be appropriate. The above reported findings add to the evidence for the role of omega-3 fatty acids as anti-inflammatory nutrients. A given, of course, is the inflammatory nature of chronic diseases such as rheumatoid arthritis. However, in the recent literature, the inflammatory aspects that contribute to the development of atherosclerosis have been readdressed and discussed as investigators seek molecular mechanisms to explain this chronic disease. Two papers that were published concurrently or after the workshop should be considered, also, as they add to the evidence for the anti-inflammatory actions of omega-3 fatty acid. Investigators used the cholesterol-fed rabbit as the model to study the effects of omega-3 fatty acid on coronary occlusion-reperfusion (6). Their results point to a reduced production of free radical generation during the short term occlusion-reperfusion experiments in rabbits fed omega-3 fatty acid. In a retrospective study, In 1994, Shahar et al. (7) demonstrated that a high dietary intake of omega-3 fatty acid may protect smokers from chronic obstructive pulmonary disease (bronchitis and emphysema). The authors suggest that omega-3 fatty acids may be acting by interfering with neutrophil activation/accumulation in the lungs of smokers. Such a mechanism may be related to a combination of reduced chemotaxis, lower production of leukotriene B_4, and decreased production of reactive oxygen intermediates. The actions of omega-3 fatty acid on activation of leukocytes parallel these seen in platelets. These omega-3 fatty acids function to inhibit or slow down the interactions among platelets, neutrophils/monocytes, and blood vessel surfaces and thus can be characterized as anti-thrombotic and anti-atherogenic by still undefined mechanisms. Some of the actions of omega-3 fatty acids may be independent of eicosanoid production by affecting signal transduction networks distal to the actions of these lipid mediators. Recently, I have shown that platelets from rats fed fish oil produce less reactive oxygen intermediates when stimulated with collagen compared to platelets from rats fed a omega-6 fatty acid-rich diet (8). Dietary fish oil reduces the collagen-stimulated oxidative burst in rat platelets. The production of reactive oxygen species (measured as H_2O_2 flurometrically in conjunction with flow cytometry of whole blood samples) was not inhibited by the addition of indomethacin and, thus, appears to be independent of thromboxane A_2 production.

There is another cell signal whose generation may be modulated by ingestion of omega-3 fatty

acids and that may be either dependent or independent of eicosanoid synthesis. This signal, ionized calcium, plays a central role in the intracellular networks that control cellular responses initiated by ligand-membrane receptor interactions. For example, platelets from patients with severe arteriosclerosis appear to have defects in the mechanisms that control the amount of intracellular ionized calcium resulting in significantly higher resting levels of this signal. These elevations under basal conditions are probably a reflection of a chronic activation in vivo (9). We have recently shown that platelets from rats fed fish oil have a lower basal flux of ionized calcium across the plasma membrane compared to platelets from rats fed corn oil (8). This diminished flux was demonstrated by monitoring the decay of the ionized calcium probe, aequorin (a bioluminescent protein). In the same experiment, using chlortetracycline as a probe for calcium in intracellular membranes, we also demonstrated less thrombin-stimulated release of ionized calcium in platelets containing omega-3 fatty acid addition. These results contribute to evidence for effects of dietary omega-3 fatty acids on the generation of the ionized calcium signal.

In addition to effects on calcium homeostasis in platelets, there are reports that demonstrate a protective effect of omega-3 fatty acids on cardiac glycoside-induced toxic levels of intracellular ionized calcium (10, 11). This protection was shown to arise from a modulatory effect of omega-3 fatty acids on the operation of membrane calcium channels. Pertinent to the above is the work showing that dietary fish oils prevent ventricular fibrillation during occlusion-reperfusion in rats and monkeys (12). Consideration of mechanisms discussed above may help to explain the results of the secondary trial that demonstrated reduced mortality after consuming several fish meals per week (13).

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SYNOPSIS: Recent Progress In Omega-3 Fatty Acid Cancer Research

David P. Rose, American Health Foundation

In general, whereas omega-6 polyunsaturated fatty acids enhance experimental carcinogenesis, and stimulate the growth of transplantable tumors, fish oils rich in omega-3 fatty acids exert suppressive effects. Thus, the omega-3 fatty acids inhibit the development of chemically-induced rat mammary tumors, and exhibit similar activity in a pancreatic cancer and a colon cancer model (Karmali, Biological Effects and Nutritional Essentiality, 351, 1988).

However, a review of the current status of research on dietary omega-3 fatty acids and cancer indicates that a number of problem areas exist which require attention before proceeding to the development of clinical trials (Table 1). Although we have focused on breast cancer, our own research area, the following discussion is equally applicable to other tumor types.

(1) **Dose-Response to Dietary Fish Oils in Experimental Cancer Studies.** A study by Cohen et al. (Lipids 28:449, 1993) failed to demonstrate a dose-related protective effect of dietary menhaden oil on N-nitrosomethylurea-induced rat mammary carcinogenesis. Similarly, we found that a diet containing 11.5% menhaden oil and 11.5% corn oil was equally effective as 18% menhaden oil and 5% corn oil in suppressing the growth of a human breast cancer cell line in the mammary fat pads of nude mice (Rose and Connolly, JNCI 85:1743, 1993).

(2) **The Role of Lipid Peroxidation.** This is a critical issue given the concerns about peroxidation products as a health hazard. Recent publications by Gonzalez et al. (Carcinogenesis 12:1231, 1991; Lipids 28:827, 1993) showed that the suppressive effect of dietary fish oil on the growth of human breast cancer cell lines in nude mice was lost when high levels of antioxidants (DL tocopherol acetate, 8g/kg; TBHQ, 4g/kg) were also incorporated into the diets.

We have found that the Fish Oil Test Material Program vacuum deodorized fish oil, containing 1 g/kg each of alpha- and gamma-tocopherol and 0.2 g/kg TBHQ, and menhaden oil without supplemental antioxidants were equally effective in inhibiting the growth of MDA-MB-435 human breast cancer cells in the mammary fat pads of nude mice when fed at 18% (wt/wt) plus 5% corn oil, but the suppressive effect on lung metastasis was lost in the presence of the antioxidants. One approach to this problem might be to evaluate alpha-linolenic acid in the nude mouse models because this short-chain omega-3 fatty acid is considerably less susceptible to peroxidation than the long-chain EPA and DHA (Cho et al., JAOCS 64:876, 1987).

(3) **The Specificity of EPA and DHA as the Effectors of Tumor Growth and Metastasis Suppression by Fish Oils.** There has been considerable debate as to whether the reported beneficial effects of dietary fish oil on tumor development and progression are entirely, or principally, due to EPA and DHA or whether minor constituents have a role. This is far from being an academic point, because (a) biologically effective minor components might be lost during a partial purification, and (b) the use of a pure omega-3 fatty acid has advantages when animal studies are translated to clinical trials.

We have addressed this issue, and shown the pure EPA and DHA (as the ethyl esters) are highly effective in our nude mouse-human breast cancer cell model (Rose et al., JNCI 87:587, 1995). Both omega-3 fatty acids were significantly more effective than fish oil in inhibiting tumor growth and metastasis; at 4% (wt/wt) EPA was superior to DHA in suppressing the development of lung metastases. It should be noted that whereas 3 g/kg of alpha-tocopherol was incorporated into the omega-3 fatty acid preparations, the fish oil did not contain added antioxidant.

(4) The Translation of Experimental Studies to the Clinic.

Clinical studies of omega-3 fatty acids thus far reported show promise in the prevention of colon and breast cancer. The effect of fish oil dietary supplementation on rectal mucosal cell proliferation has been examined; inhibition of accelerated growth was demonstrated in individuals at high risk for colon cancer, and also retardation of the mitotic rate in healthy volunteers (Anti et al., Gastroenterology 103:883, 1992; Bartram et al., Gastroenterology 105:1317, 1993; Bartoli et al., Mol. Aspects of Med. 14:247, 1993). Dietary intervention with omega-3 fatty acids also normalized leukocyte adenosine diphosphate ribosyl transferase (ADPRT) activity, a biomarker of high cancer risk (Rowsch et al., Nutr. Cancer 16:197, 1991), and similar results were obtained in a study of 16-alpha hydroxylated estrogen, a putative marker for increased breast cancer risk (Osborne et al., Cancer Invest. 6:629, 1988).

In breast cancer, two key issues are the patient populations to be included in the first generation of clinical trials, and the development of suitable intermediate biomarkers of therapeutic response.

A trial of dietary omega-3 fatty acids as agents for the primary prevention of breast cancer may be rejected on the grounds of feasibility, expense, and competition with on-going studies (tamoxifen prevention trial and the Women's Health Initiative Trial). However, serious consideration should be given to working towards a trial of omega-3 fatty acids as part of an adjuvant therapy regimen. It should be noted that while an NCI-supported low-fat intervention trial is underway, this is confined to postmenopausal patients; any therapeutic benefit of omega-3 fatty acids should apply equally to premenopausal women.

A second area ripe for clinical investigation is the potential benefit of omega-3 fatty acids in the cachectic cancer patient (Tisdale, Prostaglandins Leukotrienes Essential Fatty Acids 48:105, 1993; Dagnelie et al., Lipids 29:195, 1994), although tumor types other than breast would be appropriate (e.g. pancreas, lung) since cachexia is not usually a feature of advanced breast cancer.

Before giving consideration to a dietary omega-3 fatty acid intervention trial in post-surgically resected breast cancer patients, additional support for the concept may be obtained from further application of the nude mouse-human breast cancer cell model. Of particular relevance is a currently on-going study (D.P. Rose and J.M. Connolly) in which dietary EPA and DHA supplementation is being commenced after the excision of mammary fat pad tumors; the end-points here are local recurrence and the emergence of lung metastases.

Comments and Conclusions

(i) Most animal studies have been done under extreme dietary conditions: i.e. high total fat intake with either very high or very low ratios of omega-6 to omega-3 fatty acids. In consequence, there is often difficulty in discerning their relevance to the human situation. There is a need for careful full-range dose-response animal studies which incorporate fat intakes and proportions that are likely to be attainable in a clinical setting.

(ii) There is a critical need for studies which are directed at understanding the modifying influences of antioxidants, vitamins, and trace metals (e.g. iron, selenium) on tumor responses to omega-3 fatty acids.

(iii) Appropriate biomarkers of response to dietary omega-3 fatty acids need to be developed. Ideally, these would be intermediate therapeutic response markers, e.g. 16 alpha-hydroxylated estrogen suppression in breast cancer, thymidine index, inhibition of type IV collagenase activity (in serum and tissue).

(iv) An Interdisciplinary Group approach should be encouraged; the interactive research project grant mechanism seems particularly appropriate.

(v) THE FISH OIL TEST MATERIALS PROGRAM has been a reliable source of test materials for the scientific community. This Program supports studies in basic and clinical research on a fair and meritocratic basis. Investigators have been provided with materials that have been subjected to stringent quality assurance testing. The staff in Charleston have provided scientific guidance in the conduct of the studies and have carefully monitored the progress of the various studies. The Program has been flexible in helping investigators with special research needs for n-3 PUFAs. Use of standardized fish oil products has replaced the use of random, untested fish oil samples often obtained from various industry sources. As a result, the standard of research is elevated resulting in more accurate and uniform findings in the omega-3 fatty acid field.

There is a definite need for the pure omega-3 fatty acids (EPA, DHA and, perhaps, alpha-linolenic acid) to be made available in bulk quantities, and both with and without the addition of antioxidants. This valuable resource would permit investigators to perform the necessary nude mouse experiments noted in (ii) above, and also set the stage for clinical intervention trials (for which capsule preparations would be required).

TABLE 1: PROBLEM AREAS IN FISH OIL AND CANCER RESEARCH

1. Is there a "dose-response" in the suppressive effects of dietary fish oil on carcinogenesis and tumor progression?

2. Do fish oils exert their effects primarily through the formation of lipid peroxidation products, and if so what are the implications in terms of clinical applicability?

3. Are the effects of fish oils attributable entirely to the principal omega-3 fatty acids (EPA and DHA) present, or are minor constituents active?

4. Where do we start in designing clinical trials?
   -Prevention?
   -Adjuvant nutritional therapy?
   -Cachexia in advanced disease?

5. What intermediate biomarkers of response to omega-3 fatty acid administration are available (or need to be developed) for clinical trials?

DISCUSSION COMMENTS: Recent Progress In Omega-3 Fatty Acid Cancer Research

Dr. Rashida Karmali

A review of the current status of research on polyunsaturated fatty acids (PUFAs) of the omega-6 and omega-3 series indicates disparate effects of these classes of PUFAs in experimental and human cancers. Omega-3 PUFAs exhibit protective effects on the development of carcinogen-induced tumors, on the growth of solid tumors and on metastatic disease in experimental models. The various mechanisms of action proposed involve a competitive inhibition of omega-6 PUFA by omega-3 PUFA.

Experimental Cancer

Omega-3 PUFAs inhibit the development of two carcinogen-induced mammary tumors, one carcinogen-induced pancreatic tumor model, and one carcinogen-induced colon cancer tumor. In addition, omega-3 PUFAs have a protective effect on transplanted solid mammary, prostatic and sarcoma tumors and suppress metastases in two metastatic mammary tumor models.[1-2]

Eicosapentaenoic acid (EPA), an omega-3 PUFA, inhibits cachexia and tumor growth in a murine colonic adenocarcinoma model. This anticachectic effect of EPA appears to be due to an inhibition of the action of tumor-produced catabolic factors at the level of the adipocyte and protein degradation in the skeletal muscle. These findings have led to in clinical trials of EPA in cancer patients with cachexia.[3]

Some of the metabolic and biochemical studies in experimental cancers include establishment of optimum dosage levels of omega-3 PUFAs, evaluation of the role of anti-oxidants and peroxidation products, and modulation of biomarkers and oncogenes. It is hoped that these studies will further our understanding of the underlying mechanisms and ultimately lead to large scale trials in humans.

The Translation of Experimental Studies to the Clinic

Epidemiological studies in the Greenland Eskimos and the Japanese suggest that incidence of breast, colon and prostate cancer was very low but increased during a period of westernization of foods resulting in a lower intake of fish and higher consumption of saturated fat and omega-6 PUFA.[4]

Before extending the research findings which were made in experimental cancer models to human clinical trials, two key issues must be addressed: (i) the development of suitable biomarkers for different types of cancers, and (ii) the patient populations to be included in the first round of trials.

Clinical studies on omega-3 PUFAs thus far reported are promising for breast and colon cancers. The effect of omega-3 PUFAs on rectal cell proliferation was studied in subjects at risk for colon cancer and in healthy volunteers. Results obtained from these studies suggest that omega-

3 PUFAs may protect high-risk subjects from colon cancer.

In addition, omega-3 PUFA intervention for six weeks normalized the activity of a biomarker for risk, adenosine diphosphate ribosyl transferase (ADPRT) in white cells taken from women at high risk for breast cancer. [6] Similar results were obtained upon monitoring 16-alpha hydroxylated estrogen. These preliminary studies are encouraging in reference to making the feasibility of large-scale trials to become a reality

An additional area ripe for clinical investigation is the potential benefit of EPA in cachectic cancer patients. Serious consideration should also be given to trials of omega-3 PUFA in post-surgically resected breast cancer patients based on findings of anti-metastatic actions in experimental mammary cancer models.

The FISH OIL TEST MATERIALS PROGRAM has been a reliable source of test materials for the scientific community. This Program supports studies in basic and clinical research on a fair and meritocratic basis. Investigators have been provided with materials that have been subjected to stringent quality assurance testing. The staff in Charleston has provided scientific guidance in the conduct of the studies and have carefully monitored the progress of the various studies. The Program has been flexible in helping investigators with special research needs for omega-3 PUFAs. Use of standardized fish oil products has replaced the use of random, untested fish oil samples often obtained from various industry sources. As a result, the standard of research is elevated, resulting in more accurate and uniform findings in the omega-3 fatty acid field.

In summary, promising results both in experimental and human studies have been reported on the chemopreventive actions of omega-3 fatty acids in cancer. Continued support to the investigators from the FISH OIL TEST MATERIALS PROGRAM will hopefully keep the momentum in research and guarantee a high quality of research in the future.

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SYNOPSIS: Potential Application of omega-3 Fatty Acids in the Treatment of Diabetes Mellitus

John D. Bagdade, M.D.

Despite the fact that omega-3 fatty acids have an expanding spectrum of actions that are anti-atherogenic, there has been no coherent effort to date to perform a clinical trial to determine whether they might benefit populations at risk. I wish to review the impact that omega-3 fatty acids have on a number of cardio-vascular risk factors unique to diabetic patient. The positive effects of omega-3 fatty acids are of sufficient magnitude, in my opinion, to warrant a clinical trial. Indeed, if a trial on the efficacy of omega-3 fatty acids to retard the development of atherosclerosis were to be performed, the diabetic population, particularly those patients with NIDDM (non-insulin dependent diabetes mellitus), would appear to be the ideal group to study because many omega-3 fatty acid-responsive risk factors are over-represented in NIDDM.

One of the enigmas in attempting to understand cardiovascular disease in diabetics is the fact that only about 25% of their morbidity and mortality from macrovascular complications can be attributed to conventional risk factors. This fact has led to the identification of several new risk factors specific to diabetic vascular disease. These include the long-term adverse effects of glyceration of tissue proteins, alterations in hemostasis, insulin resistance, hyperinsulinemia, altered interactions between humoral-producing arterial wall cells, and disturbances in the composition and function of lipoproteins that enhance their atherogenicity ("diabetic dyslipidemia")(1). There is much to support the position expressed by Lloyd Axelrod that omega-3 fatty acids are "... a gift from the sea" for diabetics since they have the capacity to reverse many of these abnormalities (2).

The literature regarding the use of omega-3 fatty acid in NIDDM is confusing, largely because many of the studies performed have varied in the type and composition of the marine lipid employed, the level of dietary control and duration of study, and the type of patient included. For these reasons, it has been unclear whether the various omega-3 fatty acid preparations employed were potentially beneficial or even harmful. Careful extended controlled metabolic studies by Dr. William Connor and his colleagues at the Oregon Health Sciences University have established that glycemic control is not adversely affected and plasma lipid concentrations uniformly improve in NIDDM patients receiving oral antidiabetic therapy treated for six months with omega-3 fatty acids (3). This critical dietary study dispels two of the major concerns raised earlier (e.g., deterioration of glucose tolerance and increased LDL) which have been responsible for the decline in interest in omega-3 fatty acids in recent years.

In fact omega-3 fatty acids have salutary effects on several of the more recently recognized processes that are believed to be important in atherogenesis. Fish oils for example reverse the procoagulant state often present in NIDDM patients by decreasing thromboxane-2 production, platelet aggregation, and plasma and blood viscosity (4). While there is no good evidence that omega-3 fatty acid treatment alters the degree of insulin resistance present in NIDDM, the evidence is that it does not worsen and peripheral glucose uptake is unchanged. The fact that fish oils have no net effect of glycemia (5) suggest that it will not affect the glycation process.

Since omega-3 fatty acids decrease hepatic synthesis and secretion of triglyceride-rich

lipoproteins, the dyslipidemia of NIDDM which is most often manifested by increases in the plasma triglyceride (TG) concentration would seem to be a good target for intervention. Indeed, Connor et al. (3) have shown that VLDL levels uniformly decline in NIDDM subjects consuming dietary sources of marine lipids and LDL levels fall This effect on LDL also is contrary to what has been reported earlier by others in less well controlled studies.

The question of whether lipoproteins from omega-3 fatty acid ingesting populations are more susceptible to oxidative stress *in vivo* is an important but still unresolved issue. If the resistance of lipoproteins to oxidative stress is reduced, then the potential atherogenicity of the plasma lipoproteins would be increased. However, just because omega-3 fatty acid-enriched LDL, for example, is more susceptible to in vitro oxidation does not indicate that it is *in vivo*. It appears that when the intake of fish oils is high and the *potential* risk of oxidation of the omega-3 fatty acids is increased, there is a compensatory increase in free-radical scavenging systems such as glutathione peroxidase(6). If this were not the case, then the fish-eating populations that have been the object of such intense epidemiological interest would be expected to have higher rather than lower cardiovascular risk.

When omega-3 fatty acids are incorporated into lipoprotein lipids, their presence alters their composition and function. These effects appear to be most apparent in certain key steps in reverse cholesterol transport. One effect of omega-3 fatty acids is to decrease cholesterol esterification(7). Another process of emerging importance is the impact on the activity of cholesteryl ester transfer protein (CETP). Since cardiovascular risk appears to be increased when CETP activity is elevated (8), the normalization of CET by omega-3 fatty acids is another metabolic effect that is apparently antiatherogenic (9). Preliminary evidence also indicates that fish oils also have a favorable effect on the behavior of arterial wall cells. Cytokine (interleukin-1; tumor necrosis factor) production by macrophages (10) and PDGF by arterial wall smooth muscle cells are reduced and nitrous oxide by endothelial cells is increased by omega-3 fatty acids (11).

CONCLUSIONS

1. Omega-3 fatty acids have a wide spectrum of antiatherogenic actions.
2. The beneficial effects of omega-3 fatty acids on blood pressure, coagulation, and lipoprotein transport could be useful in patients with NIDDM.
3. Carefully performed short and long-term metabolic studies in diabetic patients demonstrate that omega-3 fatty acids can be used therapeutically without deterioration of glycemic control which has been a concern that has restricted their use.
4. It is probable that the increase in LDL-C observed in some patients following ingestion of some omega-3 fatty acid preparations is likely of no clinical consequence because of the pervasive changes in the composition, physical properties, and function of lipoproteins that result from treatment.
5. Because of the tremendous costs that result from diabetic macrovascular complications and what is now unequivocal evidence that omega-3 fatty acids can ameliorate a number of cardiovascular risk factors present in NIDDM, it is timely that a controlled clinical trial be undertaken to assess whether omega-3 fatty acid treatment can reduce morbidity and mortality in this high risk population.

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DISCUSSION COMMENTS: Omega-3 Fatty Acids and Diabetes Mellitus

William E. Connor, M.D.

Dr. Bagdade has appropriately stressed the potential importance of the omega-3 fatty acids from fish oil in patients with diabetes mellitus. These patients have an exaggerated incidence and death rate from vascular disease, including stroke, coronary heart disease and peripheral vascular disease. There is good evidence that EPA and DHA provided in fish oil have many beneficial effects in diabetic patients as they do in non diabetics. These beneficial effects include the following:

1. Hypertension - lowers blood pressure
2. Anti-thrombotic
3. Experimental animal studies to inhibit the growth of atherosclerotic plaques
4. Antiarrhythmic actions
5. Lowers plasma lipids and lipoproteins
6. Prevents coronary heart disease

This being said, an important question to be answered is will fish oil affect diabetic control adversely? There seems to be no disagreement that the use of fish oil in juvenile or type I diabetics does not cause a deterioration in glucose homeostasis. It is in the type II, adult onset diabetic patients that there still is some controversy, although a recent review by Heine in the New York Academy of Sciences concluded that there was little evidence that diabetic control was worsened by fish oil. Our experience has been in a one year crossover study comparing the effects fish oil and olive oil administered randomly each for a six month period of time upon diabetic control in type II diabetic patients. Diabetic control as measured by the usual parameters was unchanged by fish oil verses olive oil. One of the problems in the past has been the imposition of the additional calories provided by fish oil without a concurrent reduction in the other fat in the diet. This has led to a hypercaloric state which has produced weight gain and deterioration of glucose homeostasis. If the caloric content is balanced, as it was in our study, then there is no theoretical reason to suspect that fish oil would disturb diabetic control. Such was certainly the case in the longest study carried out to date for a period of one year.

I certainly concur with Dr. Bagdade's suggestion that the time is ripe for clinical trials of omega-3 fatty acids in diabetic patients.

SYNOPSIS: Inflammation/Cytokines and Omega-3 PUFA

Dr. Richard I. Sperling, M.D.

The focus of the talk was on the effects of omega-3 PUFA on the activity of inflammatory diseases, on the effects on cell membrane receptors and on cytokine formation. I will touch just briefly on the latter topic as Dr. Robinson will address this issue in greater detail.

The effects of dietary omega-3 PUFA on disease activity in patients with active rheumatoid arthritis (RA)

Several small clinical studies of the effects of low dose dietary omega-3 PUFA supplementation in patients with active RA suggest modest improvement in disease activity. Larger, well-designed, multicenter trials are needed to determine the role of dietary omega-3 PUFA supplementation in the treatment of RA, but have not been carried out to date due to the high cost of such trials and the lack of funding. Paul Fortin, members of the Multipurpose Arthritis Center at Brigham and Women's Hospital, and I have analyzed the data from these completed studies using the techniques of meta-analysis and meta-analysis.

Studies were identified by a Medline literature search, by checking references of these studies, and by contacting researchers in the field. As part of the mega-analysis, eleven studies met our inclusion criteria: (1) the trial is double-blind, placebo-controlled and employs some randomization procedure; (2) parallel or cross-over design; (3) the daily dosage of EPA is greater than or equal to 1.8 gm; and has a duration of at least 6 weeks; (4) background antirheumatic medications remained constant throughout the trial; and (5) at least one of our outcome variables of interest was assessed. Data from 395 patients in 9 trials met criteria for entry into the meta-analysis. The actual sample size pertaining to each of the calculations varied. Since we started these studies, two additional trials have been completed, and another small trial is published in Spanish. Both show a modest effect of fish oil in RA.

As part of the meta-analysis, the methods and results sections for each publication were blinded and quality scores for the methods and results were determined using the criteria of Chalmers. Both the methods and results of each of these studies were rated of "moderate quality" by the independent observers. Data regarding several outcome variables (tender/painful joint count, swollen joint count, morning stiffness, grip strength, physician's and patient's global assessment, patient's global or pain assessment on a visual analog scale, and erythrocyte sedimentation rate) were abstracted onto standardized data collection forms from the primary data of the original trials by the principal investigator of the original studies. The demographic data for the treatment and placebo groups are similar.

For the traditional meta-analysis, first, a Chi-square test of homogeneity was performed for each outcome variable. Then rate differences and their standard deviations were calculated between the treatment and the placebo group for each outcome for three months minus baseline. Lastly, pooled rate differences with 95% confidence intervals were calculated using the DerSimonian and Laird method. The meta-analysis revealed significant improvements in the tender/painful joint

count and AM stiffness in the fish oil supplemented group as compared with the control oil group after three months of treatment. Improvements observed in the other five outcome variables did not reach statistical significance.

To verify the results of the meta-analysis, we also performed what we termed a "mega-analysis" based on the primary data as abstracted by the original investigators. When the primary data were tested for homogeneity, it became clear that the patient population of one study differed demographically from those of the other studies. As the results of the meta-analysis and mega-analysis did not change with the inclusion or exclusion of this study, this study was included in the analyses presented.

Unfortunately, one of the most obvious conclusions one will draw from these data set for the meta-analysis is the lack of uniformity in the quantitation of outcome measures. This was evident and troublesome for tender/painful joint count and swollen joint count as well as the corresponding indices. We attempted to overcome the limitations imposed by lack of uniformity in the quantitation of outcome measures by analyzing the data several ways in the meta-analysis. First, univariate analyses were carried out on the demographic variables at baseline individually for the raw data of each of the seven outcome measures, testing for significant differences between the control and the treatment groups. Exploratory linear and logistic regression models were performed using demographic variables, sites, treatment, and the baseline value of the outcome as predictors. Next, multivariable linear regression models were built. The results from the mega-analysis, as in the meta-analysis, show significant improvements in the tender/painful joint count and AM stiffness in the fish oil supplemented group as compared with the control oil group after three months of treatment. Again, improvements in the other five outcome variables of the treatment group relative to the control group, did not reach statistical significance. Depending on the assumptions of the model for the meta-analysis and on whether interaction terms (which never reached statistical significance) were included, the P-values for AM stiffness varied a bit and were often greater than 0.05.

The results for the meta-analysis of tender/painful joint count were then calculated. For tender/painful joint count and the swollen joint count, the primary data were handled in four different ways. Fortunately, the results obtained from each of the methods of treating the data revealed significant improvement for tender/painful joint count, and none yielded significant results for swollen joint count.

The results from the meta- and mega-analysis studies analyses show that the tender/painful joint count and AM stiffness improve significantly after three months of low-dose fish oil supplementation as compared with control oil supplementation. I believe that a large, multicenter trial is warranted to evaluate the role of omega-3 PUFA supplementation in the treatment of RA. As even high dose dietary fish oil is virtually without side effects, clinical trials of high dose fish oil therapy would be interesting.

The effects of dietary omega-3 PUFA in other disorders.

Dr. Richard Lawrence and colleagues reported in the *Lancet* last year that dietary omega-3

PUFA, providing 2.7 gm EPA daily led to significant improvements in sputum production, FEV1, FVC, and the Swatchman's clinical score in EPA-treated patients with cystic fibrosis as compared with the placebo-control group. This group also reported depression in baseline neutrophil responsiveness and a paradoxical effect of omega-3 PUFA - the augmentation of neutrophil chemotactic responsiveness, as we have observed in RA.

Recently, three small placebo-controlled trials of dietary omega-3 PUFA in active ulcerative colitis and one in inflammatory bowel disease each showed modest benefit of omega-3 PUFA in terms of improved mucosal histology, disease activity scores and prednisone requirements.

Recent work on the mechanism of anti-inflammatory effects of omega-3 PUFA .

We observed in our first study that dietary omega-3 fatty acids not only inhibited the formation of LTB_4, but surprisingly also inhibited neutrophil responses to LTB_4 and to the chemotactic peptide FMLP as well. Terano et al. observed subsequently that 4 gm daily of dietary EPA ethyl ester resulted in a significant suppression in neutrophil chemotaxis to LTB_4 and FMLP, however, DHA ethyl ester had a minimal effect on chemotaxis which was consistent with the rise in neutrophil levels EPA induced by the DHA supplement. The mechanism(s) by which dietary omega-3 PUFAs modulate transmembrane stimulation of neutrophils by chemotactic ligands were not addressed in previous studies.

Early events in the signal transduction after the binding of the neutrophil chemotactic ligands LTB4, PAF and FMLP to their respective receptors are believed to involve the activation of a G-protein which then activates an associated phosphatidylinositol-selective phospholipase C. The activated PLC hydrolyzes phosphatidylinositol-4,5-bis-phosphate ($PIP_{2)}$ resulting in the formation of inositol-1,4,5-tri-phosphate- ($IP_{3)}$ and diacylglycerols. IP3 binds to specific receptors on the endoplasmic reticulum resulting in the release of intracellular stores of calcium and activation of phospholipases A2 and D. It is also possible that the receptor-ligand complex may activate PLD and/or PLA2 directly through a G-protein. The diacylglycerols, in conjunction with the increased cytoplasmic calcium ion concentration and the activity of protein kinase C lead to activation of other specific cellular pathways, including the increased polarization of leukocytes, the assembly of elements of the cytoskeleton and cell contraction.

We have investigated the effects of omega-3 PUFA supplementation for 10 weeks with 20 g SuperEPA daily, providing 9 g of EPA and 5 g DHA in 8 healthy volunteers. Neutrophils and monocytes were isolated for biochemical investigations 4 weeks prior to starting the dietary supplementation and after 3 and 10 weeks of dietary omega-3 PUFA supplementation.

Increases in the EPA contents of all the major phospholipid classes and subclasses of neutrophils were observed after 3 and 10 weeks of dietary omega-3 PUFA supplementation. There were no significant differences in the fatty acid contents of neutrophil phospholipids observed between the 3 and 10 week. Although the change in the EPA content of phosphatidylinositol pool, of approximately 2 mole%, was much smaller than that observed in the other phospholipds, this change was highly significant $p<0.0001$. The arachidonic acid contents of the major phospholipid classes and subclasses of neutrophils, with the exception of the PI pool, declined substantially after 3 and 10 weeks of dietary omega-3 PUFA supplementation. The LTB4 and PAF receptor affinity

and number on neutrophils did not change significantly with dietary omega-3 PUFA supplementation.

The generation of inositol phosphates by neutrophils activated with 1 μM LTB4 or PAF for 15 seconds was quantitated in [^{3}H]inositol labelled neutrophils before and after 3 and 10 weeks of dietary omega-3 PUFA supplementation by on-line β-scintillation counting after product resolution by anion-exchange HPLC. The mean for all eight subjects of net inositol phosphate formation by LTB4- and PAF-stimulated neutrophils after 3 and 10 weeks of dietary omega-3 PUFA supplementation was profoundly inhibited. Net formation of inositol mono-, di- and tri-phosphate in neutrophils stimulated with either agonist each showed significant and dramatic decreases. Despite the minimal incorporation of EPA into the neutrophil phosphatidylinositol pool, formation of IP_3 by both LTB4 and PAF-stimulated neutrophils had significant negative correlations by subject and time point with the EPA contents of the respective PI pools. Dietary omega-3 PUFA supplementation did not affect neutrophil diglyceride formation under these conditions, most would be derived from the PLD pathway. Neutrophil chemotaxis to concentrations of LTB4 and PAF was assessed ex *vivo* in Boyden microchambers prior to and after 3 and 10 weeks of dietary omega-3 PUFA supplementation. Chemotaxis to each agonist was significantly inhibited after 3 weeks of dietary omega-3 PUFA supplementation, and a further significant inhibition was observed after 10 weeks. Neutrophil chemotaxis to each of the ligands correlated significantly with IP3 formation as determined for <u>each</u> subject at <u>each</u> time point, and also correlated <u>negatively</u> with the EPA contents of the PI and other phospholipids pools of the neutrophils of the respective subjects.

The inhibition of chemotaxin-stimulated IP3 formation in the absence of an effect on the number or affinity of the respective chemotaxin receptors indicates that dietary omega-3 PUFA supplementation inhibits neutrophil chemotaxis through a post-receptor inhibition of the LTB4 and PAF activation signals generated by the phosphatidyl inositol-selective PLC somewhere between the receptor and PLC.

There are several other studies which suggest that omega-3 PUFA may alter the function and signaling of cell surface receptors. Medini et al. (1) reported that dietary omega-3 PUFA also inhibits thrombin-stimulated inositol phosphate formation by rabbit platelets without measurably affecting thromboxane formation, which is an aspect of thrombin activation of platelets. *In vitro* preincubation of aspirin-treated rabbit platelets with EPA inhibits the formation of inositol phosphates, including IP3 upon stimulation with the thromboxane A_2 receptor agonist U46619. Subsequent studies demonstrated that EPA and DHA selectively decrease U46619 binding by platelet membranes thus decreasing IP3 formation, without affecting the binding of yohimbine by the platelet α-adrenergic receptor. This suggests a mechanism for omega-3 PUFA-induced impairment of TxA_2- and thrombin-mediated platelet aggregation. Lamers et al. demonstrated that *in vitro* incubation of rat ventricular myocytes with EPA or DHA decreased the inositol phosphate response upon stimulation of the α-adrenergic receptors. The effect on receptor-ligand binding was not evaluated. Weber et al. (2) have suggested that *in vitro* preincubation of retinoic acid-differentiated U937 cells with DHA blunts the rise in intracellular calcium ion concentration upon PAF- and LTD4-stimulation through a noncyclooxgenase-mediated oxidation product of DHA. These studies demonstrate that omega-3 PUFA dramatically reduce cellular signaling and responses via certain receptors.

In 1989, Endres et al. (3) reported the effects of six weeks of dietary supplementation with

omega-3 PUFA on cytokine formation by LPS-stimulated peripheral blood mononuclear cells. They found a minimal effect after the 6 weeks, but significant inhibition in LPS-stimulated IL-1α, IL-1β and TNF formation was observed 10 weeks after discontinuing the supplement. While some studies have contradicted the findings regarding IL-1, most studies have supported the finding, including in omega-3 PUFA treated patients with RA. Most studies have not reproduced the findings regarding TNF, and in fact some studies show that omega-3 PUFAs increase TNF formation. Dr. Robinson will address the effects of omega-3 PUFA on cytokine production in his summary. There are two points I would like to mention, however. The first is that LTB_4 and PAF alone are weak stimulators of monocyte/macrophage IL-1 and TNF production and enhance LPS-stimulated IL-1 and TNF production substantially. Thus, if omega-3 PUFA inhibit LTB_4 and PAF formation *in vivo*, then they may secondarily inhibit monocyte IL-1 and TNF production. Second, as omega-3 PUFAs effect cell signalling via some cell membrane receptors, omega-3 PUFA may possibly inhibit signals, such as those induced by LPS, leading to cytokine formation.

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SYNOPSIS: Dietary Omega-3 Fatty Acids and the Development of the Brain and Retina in Human Infants

Susan E. Carlson, Ph.D, William E. Connor, M.D., and John C. Wallingford, Ph.D.

There is an extensive literature which demonstrates that essential fatty acid deficiency (both of the omega-3 and omega-3-6 families) influences behavior (1) and retinal and visual development (2) in animals. Animals made deficient in docosahexaenoic acid (DHA) have lower concentrations of DHA in retina and brain, poorer retinal responses to light (electroretinograms or ERGs), poorer visual acuity development and different behaviors compared with controls fed omega-3 fatty acid deficient diets. ERG responses are also abnormal in preterm infants fed omega-3 fatty acid deficient diets (3).

During development, the human infant obtains omega-3 fatty acids in several ways. In intrauterine life, omega-3 fatty acids are transported from the maternal blood via the placenta into the fetal circulation where they can be utilized for membrane development in the brain and retina. The amount of omega-3 fatty acids in the diet of the mother is an important factor in how much might be transferred into the fetus. Studies in rhesus monkeys have shown low levels of DHA in the blood, the retina, and the brain of the newborn infant when the maternal diet was deficient in linolenic acid, the 18 carbon essential omega-3 fatty acid precursor of DHA (4). Recent studies have shown that omega-3 long chain polyunsaturated fatty acids (LCP) in the maternal diet influences the circulating omega-3 LCP in the newborn human infant (5). Important questions remain to be answered as to what sources and quantities of omega-3 fatty acids in the maternal diet would assure the best possible intrauterine accumulation of DHA for development of the brain and the retina.

During the period following term birth, DHA continues to accumulate (6). The natural source of omega-3 fatty acids for mammals in the interval after birth is the milk produced by the breast during lactation. Here also the quantities and kinds of omega-3 fatty acids present in the diet of the lactating woman influence the concentrations of omega-3 fatty acids in milk (7). The optimal amount of omega-3 fatty acids remains undefined. The concentration of DHA in milk specimens throughout the world shows considerable variation (8). The lowest concentrations have been reported among vegan vegetarians (9) and lactating women in the United States (10,11) and the highest among fish-eating mothers (12,13).

Should linolenic acid or preformed DHA be provided to the human infant in the event that an artificial formula feeding is utilized from birth? The answer to this question may or may not be related to the gestational age of the infant at birth. The current practice in the United States is to provide an infant formula whose fatty acid composition is derived from vegetable oils that provide linolenic acid (18:3 omega-3 fatty acid). This fatty acid is the building block for the synthesis of DHA, the predominant fatty acid in the phospholipid membrane of neurons and retinal cells. Linolenic acid is the only omega-3 fatty acid currently recognized as essential for the developing

infant by the American Academy of Pediatrics (14). However, linolenic acid may not provide for optimal neural and retinal omega-3 LCP accumulation and, therefore, for optimal visual and behavioral development in infants.

There is a precipitous drop in plasma and erythrocyte DHA after birth in infants supplied only with linolenic acid from formula regardless of whether they are born at (10,15) or before (16) term. These early reports have been confirmed repeatedly by others. Recently this decline has been associated with lower concentrations of DHA in the central nervous system compared with infants fed human milk DHA (17,18).

Preterm infants may be at higher risk than term infants for less than optimal development related to DHA accumulation in neural and retinal membranes. Three randomized trials completed to date have supplied formulas with and without DHA to preterm infants. They will be addressed in detail below. Meanwhile, a number of groups have reported different functional outcomes in breast-fed compared with formula-fed term infants. Recently there has been a tendency to attribute differences in outcome to demonstrated or postulated differences in omega-3 LCP status between these groups. However, until data from randomized trials are available, such associations should be interpreted cautiously. To date no reported study has controlled (not attempted to correct statistically for the differences after failing to control!) for socioeconomic differences between breast- and formula-fed infants.

A trial conducted in one of our centers (that of SEC) shows a pronounced effect of the education level of the parents on visual attention (19). This effect was the same as, but independent of, the effect of DHA status alone. Such data caution against failing to regard the effect of socioeconomic status on development. As randomized control trials in term infants become available, they need to be analyzed for validity with the same scrutiny as the preterm studies now available.

We wish to ask if a dietary source of omega-3 LCP to preterm infants can improve their neural development compared with linolenic acid alone. To date, only three randomized trials of omega-3 LCP supplementation have been designed to study this question in preterm infants. All have used a source of marine oil as a source of DHA. Two have also provided significant quantities of eicosapentaenoic acid (EPA, 20:5 omega-3 fatty acid) in combination with DHA. The design of each of these studies has been described in terms of population characteristics, diet composition, duration of study, planned study outcomes, and results. The results of each were evaluated for *internal validity* (the confidence with which one can infer a causal relationship between two variables in the context of the study population) and *external validity* (the extent to which one can infer that the relationship found in the study population is true for other population is true for other populations and conditions).

Internal validity. A. The results of these trials shows that preterm infants fed omega-3 LCP have higher circulating levels of DHA (20), more physiological ERG responses at 36 wk (one month before expected term delivery) (3), and better visual acuity at 2 and 4 months compared to controls (21-23). B. Dietary omega-3 LCP also influenced visual attention(19,24,26) and, in conjunction with a nutrient-enriched formula, increased 12-month scores on the Barley MDI (27). C. None of the trials completed to date have sufficient power to address safety issues, with the exception of growth addressed in two trials (28,29), or to determine if omega-3 LCP supplementation increases

or decreases the risk of specific medical problems in very-low-birth-weight infants. The issue of safety is an important one for the formula industry which fears it would singlehandedly bear the risk for adverse outcomes in preterm infants despite the fact that they frequently suffer such outcomes regardless of feeding.

External validity. Because the infants studied have been selected from among healthy, appropriately-grown very-low-birth weight infants, it cannot be concluded from these data that sicker preterm infants or more mature term infants would benefit from omega-3 LCP supplementation. In fact, data from our second randomized trial suggest that while infants with chronic lung disease do increase blood phospholipid DHA in response to DHA, they do not have early improvements in visual acuity (30), and their 12-month Barley scores are not enhanced.

Relevance to the Fish Oil Test Materials Program. Available data suggest research on omega-3 fatty acids may have a profound public health impact. How are FOTMP materials important to research on omega-3 fatty acids and the nervous system? The randomized trials that have been completed indicate the public health importance of these fatty acids in human nervous system development. A much larger clinical trial could evaluate risks as well as confirm benefits. Such a trial could also include sicker infants in sufficient numbers so that subgroup analyses could be made with good internal validity. The availability of a source of DHA will be necessary for other investigators who wish to study the unanswered questions about the optimal duration and concentration of dietary omega-3 LCP.

Unanswered questions. A. What is the optimal interval for administration of omega-3 LCP to preterm infants? B. Do infants born at term need omega-3 LCP or can they perform as well with dietary linolenic acid? C. What is the optimal amount of DHA that should be added to formula, e.g., our data show that diets with 0.15% to 0.2% DHA do not maintain circulating DHA at intrauterine levels during the interval when infants would normally be in utero? D. Will AA need to be provided with DHA to prevent adverse effects on growth if DHA needs to be provided beyond about 2 month past expected term?

A testable hypothesis of mechanism of action is needed. The single most important missing element for research in this area is to establish biologic plausibility. The cardiovascular and inflammatory functions of omega-3 fatty acids are seen in the roles of the various oxygenated metabolites, has not been established in central nervous system (CNS).

The lipid composition of nerve tissue, in particular the reciprocal relationship between DHA and 22:5 omega-3-6 fatty acid during omega-3 fatty acid deficiency, suggests that chain length may be a structural feature to which function needs to be related rather than the position of the terminal unsaturation. The ERG data show that there are differences in the extent to which different 22 carbon LCP function, and the importance of DHA may be more a matter of degree than type. The role of DHA essentiality in the nervous system should be dissociated from the issue of essentiality of omega-3 LCP in other physiologic systems, and we need to base our hypothesis on the apparent need for 22 carbon fatty acids.

The hypothesis of CNS function are not satisfying, in part because we do not have enough descriptive information for them at the biochemical level and in part because some hypotheses cannot be tested *in vivo*. For example, descriptive differences in membrane fluidity introduced by

substitution of 22:5 omega-6 for DHA would provide little basis for physiological *in vivo* and *ex vivo* tests. Experiments remain largely in the realm of model biophysical systems.

Where do we start to look for a testable mechanism of DHA action in the nervous system? DHA is diffusely located throughout the retina, concentrated in the rod outer segment disk membranes. The function of the rod cells is controlled by the replacement of DHA by 22:5 omega-6, but where in phototransduction is the impairment incurred? Is Professor Dratz correct when he suggests that the 22 carbon chain is a molecular spring that interacts directly with the receptor, restoring the receptor to its excitable state (31), or is there a decrease in G protein interaction with rhodopsin because of subtle changes in the microenvironment at the disk membrane? Either type of action could be important throughout the central nervous system. Since DHA are in rhodopsin function, perhaps there are comparable effects on the function of other receptors in the alpha-adrenergic superfamily at the synapse. This can be tested, or, as the recent findings from the elegant pharmacologic studies by Dr. Leaf and his associates suggest, DHA might affect ion channels directly (32), which is also completely consistent with empirical ERG data. Can we demonstrate proximity of DHA to ion channel proteins?

We also know that DHA is enriched in neurite growth cones (33). Does 22:5 omega-6 replace DHA in neurites of omega-3 deficient animals? We can quantify neurite outgrowth in culture. Does DHA affect rates of growth? Differences in visual attention occurring long after DHA is removed from the diet of preterm infants are consistent with the idea that structural DHA early in development (e.g., in the germinal matrix gliablasts) could define subsequent astrocyte and oligodendrocyte development. These possibilities are mentioned because they can be approached experimentally using FOTMP materials.

We have already progressed past the theory of omega-3 essentiality by demonstrating important behavioral effect in infants. The researchers who have found biophysical tools with which to address the unique functions suggested by nervous system lipid composition data are to be applauded. There is a need to find the mechanism for CNS function of omega-3 fatty acids. This is the hurdle that lies between hypotheses that are provocative and those that are generally accepted and provide the basis for public health policy.

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PARTICIPANT ADDRESSES

Ms. Beth Aurecchia
US Dept. of Commerce
NOAA/National Marine Fisheries Service
Charleston Laboratory
c/o NIAAA, NIH
Danac #4, Rm. 2
12501 Washington Avenue
Rockville, MD 20852

Dr. John Bagdade
Rush-Presbyterion
St Luke's Medical Center
1725 West Harrison Street, Suite 25C
Chicago, IL 60612-3724

Dr. Susan Carlson
Dept. of Pediatrics/Newborn Center
University of Tennessee
853 Jefferson Avenue
Memphis, TN 38163

Dr. Williiam E. Connor
Oregon Health Sciences University
Department of Medicine, L465
3181 Sam Jackson Park Road
Portland, Oregon 97201

Dr. Isabel Chen
Office of Pre-Market Approval
Food & Drug Administration
200 C Street, SW Room 1252
Washington, DC 20204

Dr. Patricia Fair
US Dept. of Commerce
NOAA/National Marine Fisheries Service
Charleston Laboratory
Charleston, SC 29412

Dr. Lawrence Harker
Emory University
1639 Woodruff Avenue, Room 1003
P.O. Box AR
Atlanta, Georgia 30322

Dr. Ronald Innerfield
Food and Drug Administration
Mail Code HFD-510
Room 14B04
5600 Fishers Lane
Rockville, MD 20857

Dr. Joseph Judd
USDA
Agriculture Research Service
Building 308, Room 214
BARC-East
Beltsville, MD 20705

Dr. Rashida Karmali
Pennie & Edmonds
1155 Ave. of the Americas
New York, NY 10036

Dr. Howard Knapp
Division of Clinical Pharm
Department of Internal Medicine
University of Iowa
Iowa City, IA 52242

Dr. William Lands
Parklawn Bldg. 16C-06
5600 Fishers Lane
Rockville, MD 20852

Dr. Alexander Leaf
Department of Preventive Medicine and Clinical Epidemiology
Harvard Medical School
Massachusetts General Hospital
Fruit Street
Boston, MA 02114

Dr. Orville Levander
USDA
Human Nutrition Research Center
Building 307, Room 220
Beltsville, MD 20705-2350

Dr. Lawrence Lin
Food & Drug Administration
Office of Pre-Market Approval
200 C Street, SW
Washington, DC 20204

Dr. Melvin M. Mathias
USDA
Cooperative State Research Service
Room 329R, Aerospace Bldg.
Washington, DC 20250-2210

Dr. Susan Pilch
Division of Cancer Prevention and Control
National Cancer Institute
Executive Plaza North, Room 212
6130 Executive Boulevard
Rockville, MD 20892

Dr. Dwight R. Robinson
Arthritis Unit
Bullfinch 165
Massachusetts General Hospital
55 Fruit Street
Boston, Massachusetts 02114

Dr. David Rose
Division of Nutrition and Endocrinology
American Health Foundation
Vallhalla, NY 10595

Dr. Norman Salem, Jr.
NIAAA, NIH
Room 2 (Flow Lab)
12501 Washington Ave.
Rockville, MD 20852

Dr. Norberta Schoene
USDA
Human Nutrition Research Center
Building 307, Room 220
Beltsville, MD 20705-2350

Dr. Artemis Simopoulos
President
The Center for Genetics, Nutrition and Health
American Association for World Health
2001 S Street, NW
Suite 530
Washington, DC 20009

Dr. Richard Sperling
Harvard Medical School
Brigham and Women's Hospital
Dept. of Rheumatology and Immunology
250 Longwood Avenue, Room 615
Boston, Massachusetts 02115

Dr. Gloria Troendle
FDA-HFD 510
Parklawn/Room 14B04
5600 Fishers Lane
Rockville, MD 20852

Dr. John C. Wallingford
Center for Food Safety and Applied Nutrition
Food and Drug Administration
FOB-8, HFS-465
200 C Street, SW
Washington, DC 20204

Dr. Momtaz Wassef
Division of Heart and Vascular Disease
4A-12, Federal Building
7550 Wisconsin Avenue
Bethesda, MD 20892

Dr. Paddy Wiesenfeld
FDA/CFSAN/OSN/DSAT
HFS-465
8301 Muirkirk Road
Laurel, MD 20708

Dr. Eugene Zimmerman
NIH/NIAID
Solar Bldg., Room 4A42
9000 Rockville Pike
Bethesda, MD 20892

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