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EDITORIAL

# "Meta-Analysis, Metaphysics and Mythology"

# Scientific and Clinical Perspective on the Controversies Regarding Vitamin E for the Prevention and Treatment of Disease in Humans

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The recent meta-analysis by Miller et al. in the Annals of Internal Medicine (2004; 142: Epub,)[1] has ignited a vociferous and polarized controversy about the clinical use of vitamin E in humans to reduce morbidity and mortality in various clinical disorders such as cardiovascular disease and cancer. It is time for scientists on both sides of this debate to accurately assess the facts regarding vitamin E. We still do not know about the efficacy, safety, clinical applications, proper dosage or type of vitamin E that should be utilized long-term by humans for prevention and treatment of disease. Epidemiological data from cross-sectional, case-controlled, prospective studies have shown a robust relationship between the consumption of antioxidant vitamins and minerals, or of foods with high concentrations of these nutrients, and reduction in the incidence of cancer and cardiovascular disease.[2,3,4,5,6,7] However, randomized, placebo-controlled, primary prevention trials using single and paired vitamins and antioxidant micronutrients consumed in relatively high doses over prolonged time periods have produced conflicting results regarding clinical benefit.[2,3,8,9,10,11,12,13]

Miller's meta-analysis of 135,967 adults from 19 studies who took vitamin E in doses of 16.5 IU to 2000 IU daily vs. placebo for at least one year was seriously flawed. There exists a selection bias, improper recognition and definition of the type of vitamin E (synthetic versus natural alpha tocopherol) and inclusion of studies that used vitamin Ein combination with other vitamins (over 50% of studies). Coupled with "statistical complexity" used in the meta-analysis, incorrect results, conclusions and recommendations have been reported. For example, 12 trials with fewer than 10 deaths were excluded from the analysis. The authors stated and assumed a priori that "….we anticipated that many small trials did not collect mortality data". This assumption may well have changed the entire results of the meta-analysis. On the other hand, this type of meta-analysis may be valid in other respects and provides some important information about the type and dose of vitamin E needed for clinical efficacy while avoiding adverse effects. It is possible that some scientists with biased views, preconceived notions and minimal scientific proof in human studies have also made some spurious health claims and ignored potential adverse effects regarding the synthetic d, l alpha-tocopherol (all RAC form), especially when administered in high doses. For example, when administered alone at high doses, all RAC vitamin E may have pro-oxidant effects,[13,14] inhibit glutathione S-transferases that are important in the detoxifi-

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cation of drugs and endogenous toxins,[15] increase bleeding tendency,[2,16,17,18,19] interfere with the metabolism of vitamin A and other vitamins[17,20] or produce other adverse effects in humans with long term administration.[20]

It should be noted that in many of the studies included in Miller's meta-analysis, the validity and safety of the supplemental vitamin E used could be suspect as has recently been reported by Consumer Labs.

The meta-analysis1 suggested that doses over 150 IU per day of alpha tocopherol were associated with increased mortality from all causes. However, below this dose the all-cause mortality was slightly, but non-significantly, lower in the primary analysis; but in the secondary analyses based on 4-way data, the pooled risk difference for the low-dosage vitamin E trials was significant at a -33 per 10,000 persons ($p = 0.021$). It was suggested in the meta-analysis that the concomitant use of other vitamins reduced all-cause mortality in both high and low dose vitamin E groups. Unfortunately, the authors do not distinguish between the synthetic and natural forms of alpha tocopherol in their analysis. The authors appear to be attempting to discredit all forms of vitamin E without expressly stating that their meta-analysis refers to only one form of the eight biologically different vitamin E's.[22] It is scientifically irresponsible to misrepresent the results of this study by implying that all forms of vitamin E at any dose may be harmful. The eight forms of vitamin E, four tocopherols (alpha, beta, gamma, delta), and four tocotrienols (alpha, beta, gamma, delta), are not inter-convertible in humans and all have different chemical structures, functions, biological activity and clinical effects.[22]

None of the 19 studies[1] included any of the other seven forms of vitamin E, and, in fact, most of them used the synthetic d, l alpha tocopherol form.[23] Alpha tocopherol is an OTC supplement available as natural d-alpha tocopherol (RRR-alpha tocopherol), which is only about 15% of total vitamin E found in food and synthetic d, l-alpha tocopherol (all RAC alpha tocopherol), which is a racemic mixture of the d and l forms of alpha tocopherol.[23,24,25] Gamma tocopherol is actually the most common form of vitamin E found in food (70%).[25]

It should be noted that of these eight stereoisomers, only one eighth of the total is RRR alpha tocopherol.[22] The other seven eighths are "counterfeit" vitamin E, where the stereoisomers do not exist in nature except in chemical synthesis.[22] This is similar to trans fats, which are due to the chemical hydrogenation (i.e., making of butter-like properties) of vegetable oil; trans fat is not found in the oil prior to the chemical process.

The d- form is the natural form of vitamin E and is more potent in its anti-inflammatory, antioxidant and cell signaling effects.[23,24] The natural d-alpha form has a potency of 1.5 IU/mg, whereas the synthetic d, l form is weaker at a potency of 1.1 IU/mg.[24,26,27] The d, l form is less expensive to produce and is commonly used in clinical trials.[26] The chemically derived d, l form of vitamin E is composed of eight stereoisomers, has minimal vitamin E activity such as anti-inflammatory, antioxidant or cell-signaling effects.[23,24,25] It may actually interfere with the biological activity of d-alpha tocopherol and reduce HDL-cholesterol in humans potentially increasing CV risk.[10] Finally, in the ATBC study this form of vitamin E increased the risk of intra-cranial hemorrhage in patients who were smokers.[2,18] All of the clinical trials on cardiovascular disease prevention that have used the natural d-alpha tocopherol have shown beneficial or neutral effects on morbidity and mortality, but NONE, and none of these trials (CHAOS, HOPE, SPACE, TAPS, HATS, ASAP) showed negative effects, regardless of the dose used.[2] Even those studies that used the all racemic d, l mixture of vitamin E (ATBC, GISSI, PPP, HPS, VEAPS) had at worst neutral results.[2] Finally, there may also be biological differences in the acetate and succinate forms of alpha tocopherol if administered intravenously, but not if given orally (Personal Communication, Barrie Tan, Ph.D.).

Thus, Miller's meta-analysis1 is suspect because it used some originally flawed studies, all of which used improper methodologies. In addition, the meta-analysis was very selective and excluded many important published vitamin E trials. If these other trials had been included, it may well have changed the results and conclusions. The meta-analysis also involved studies that enrolled primarily older adults with chronic diseases or conditions such as CVD, CHD, MI, smokers, CRF patients on hemodialysis, postmenopausal women on HRT, patients with Alzheimer's and Parkinson's disease, and excluded both older and younger healthier populations. Finally, the study assessed multi-vitamin combinations and/or other substances rather than vitamin E alone (beta carotene, selenium, vitamin C, zinc, copper, garlic, aspirin, fish oils, HRT and various pharmaceutical agents), reviewed data on only one form of vitamin E and had confounding variables that make the best statistical analysis questionable. For example, high doses of alpha tocopherol may also reduce intestinal absorption, cell membrane transport and utilization of other forms of vitamin E, especially gamma tocopherol.[22,28,29]

The hepatic alpha tocopherol transfer protein (TTP) has the greatest affinity for alpha tocopherol compared to gamma tocopherol (10-fold), and is crucial for the relative percent of transport of the various forms of vitamin E in the plasma lipoproteins.[22,30,31] Excessive intake of alpha tocopherol may reduce hepatic transport of other important forms of vitamin E.[22,30,31] This imbalance of the alpha tocopherol/gamma tocopherol levels in the plasma may have significant health consequences.[31,32] The natural abundance of vitamin E in diets suggest that the ratio of gamma/alpha tocopherol should be about 4:1.[25,31] Gamma tocopherol probably has a much more important role in human health than alpha tocopherol.[8,31] The ratio of

gamma/alpha tocopherol in plasma is a much more satisfactory index to measure compliance in clinical trials involving supplementation with alpha tocopherol.[22,33] Over 70% of the vitamin E in the human diet is gamma tocopherol, not alpha tocopherol.[25] The remaining 30% of the dietary vitamin E is 1/3 to 1/2 alpha tocopherol, 1/3 delta tocopherol, but with minimal beta tocopherol. The very basis of activity of alpha tocopherol was originally based on rodent infertility data, which may have little to do with biological activity or clinical effects in humans.[27]

The clinical use of high doses of synthetic beta-carotene has questionable safety and efficacy.[2,18] Beta-carotene has been reported to increase lung cancer in smokers or in those who consume alcohol according to the ATBC study[18] and increase mortality according to HPS.[2] It is important to remember that vitamin E, like beta carotene and other nutrients, does not work alone, but rather symphonically with its cousins.[13,34] To study one species of the family of vitamin E often results in unintended consequences such as those found in the beta carotene smokers study (ATBC).

There may be many reasons for the variable results and conclusions in the clinical trials for the role of vitamin E, other antioxidants and vitamins in the prevention and treatment of human disease. These reasons include inappropriate endpoints or their definitions, inappropriate assessment of the endpoints with clinical or laboratory tools, inappropriate adjuvant therapies, inappropriate patient types (biochemical and oxidative stress inclusion criteria, genetic polymorphisms), incorrect or ineffective type, dose, timing or combinations of vitamins or improper duration of the study.

It would be tempting to conclude that Miller's study provides definitive conclusions and recommendations, but this is not the case. His meta-analysis and the studies that were used in the meta-analysis are not statistically and methodologically sound enough to make such sweeping conclusions. I would offer these recommendations based on present data:

**One:** It would seem prudent and advisable to avoid consumption of synthetic racemic d, l- alpha tocopherol (all-RAC alpha tocopherol) at any dose, at this time, until further human clinical trials demonstrate clinical efficacy with no adverse effects.

**Two:** It would seem prudent and advisable to avoid consumption of single high doses (over 400 IU daily) of natural d-alpha tocopherol (RRR-alpha tocopherol). The level of 150 IU per day suggested as the "upper limit" at which total mortality begins to increase in the meta-analysis is probably not due to any direct toxicity of d-alpha tocopherol, but rather the absence of other tocopherols and/or tocotrienols due to reduced intestinal absorption or decreased hepatic alpha TTP incorporation.

**Three:** Use a mixed tocopherol complex with the correct balance of all four forms of tocopherols. The gamma/alpha tocopherol ratio should be approximately 4:1. The beta and delta balance should simulate the balance found in natural food as noted previously. This vitamin mixture should be taken each morning at least 12 hours before any ingestion of tocotrienols to avoid interference with intestinal absorption. More clinical studies are needed to determine the correct balance and doses.

**Four:** Use a mixed tocotrienol complex with the correct balance of tocotrienols that simulates that found in natural food. As with most other areas of nutritional science, this should be almost intuitive and is backed by some sound science. Preliminary evidence suggests that the delta and gamma tocotrienol have more biological activity and clinical benefits compared to alpha and beta tocotrienol. This vitamin mixture should be taken at night at least 12 hours after the tocopherol complex. More clinical studies are needed to determine the correct balance and doses.

**Five:** Take the mixed vitamin E complexes above with other antioxidants (vitamin C, mixed carotenoids, B-vitamins, vitamin D, CoQ-10, R- lipoic acid, etc.) to provide nutritional balance, improve total antioxidant effects, avoid potential pro-oxidant effects and enhance recycling of vitamins. Combine this vitamin regimen with 8-10 servings of fresh fruits and vegetables per day with attention to foods with good balanced vitamin E content such as soybeans and wheat germ. Objective testing to determine nutritional deficiencies, oxidative stress profile, antioxidant defenses/capacity and inflammatory status are probably advisable and necessary to prescribe an optimal nutritional and nutraceutical-based regimen for patients.

**Six:** Design and implement scientifically valid prospective clinical trials on the various forms of vitamin E to provide accurate scientific rationale to the type, dose, timing and combinations that improve morbidity and mortality in humans.

The recent SU.VI.MAX study[35] of 13,017 French adults, aged 35-60 years, is an example of a more scientifically valid trial. After 7.5 years of low dose antioxidant supplementation, the total cancer incidence and all cause mortality in men was reduced by 31% and 37% respectively ($p = 0.004$). In this study 30 mg of d-alpha tocopherol (45 IU of d-alpha tocopherol) was used in conjunction with ascorbic acid, selenium, zinc and beta carotene.

Meta-analysis, like meta-physics, is not always scientifically valid, but there may be some hidden truths for the wise observer. One should ask why in this meta-analysis the authors failed to distinguish between studies using the different forms of vitamin E prior to performing their analysis. Studies should always attempt to eliminate as many sources of bias as possible. If an adverse effect occurred only with the synthetic d, l alpha tocopherol, then combining the studies all together with both synthetic and natural vitamin E introduces an obvious source of error. Thus, including different forms of vitamin E in this meta-analysis

clearly introduces a confounding variable leading to potentially erroneous results and conclusions. A re-analysis that separates out this confounding variable should be done.

Opinions and interpretation of science are often blinded by personal prejudice and/or preconceived notions. We should all remember the wise words of Socrates at the Oracle of Delphi when asked what the definition of knowledge and wisdom were, "That which I do not know, I know that I do not know". We have much yet to learn about vitamin E and should keep our minds open as the scientific story of this contentious nutrient continues to unfold.

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EDITORIAL COMMENTARY

# Evaluating the Safety of Dietary Supplements

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In late fall of 2004 the Institute of Medicine (IOM) and National Research Council of the US National Academies published "Dietary Supplements: A Framework for Evaluating Safety." The report is a "framework for prioritizing and evaluating the safety of dietary supplements based on existing information available to the FDA and others." It does not concern itself with health maintenance effects or other related health benefits of dietary supplements.

Originally proposed by the Committee on the Framework for Evaluating the Safety of Dietary Supplements, this report in part was released in July of 2002 for comment. Six prototype safety monographs were subsequently proposed. During the fall of 2002–2003, the committee solicited input to both the framework and these monographs.

The report was primarily the responsibility of the staff of the Food and Nutrition Board (FNB) of the IOM. The FNB worked collaboratively with the Board of Life Sciences (BLS) of the Division of Earth and Life Studies of the National Research Council (NRC) to produce this report. Included are lists of expert consultants and others who contributed to it, including those teams who developed the prototype monographs. Thirteen appendices are found at the end of the report, most of which summarize information found in the monographs.

The report initially presents the charge to the committee and general background information about dietary supplements. Valuable comparative information concerning similar frameworks for evaluating the safety of other substances, which addresses issues such as pre-market approval and post-market surveillance, follow that introduction. Finally, this section concludes with a list of seven (7) attributes that guided the final framework's development.

The framework itself is discussed in detail starting with three major components: Signal Detection, Initial Review of the Signal, and Integrative Evaluation. The report diagrams how these impact the FDA's decision to take action on a supplement. Risk assessment is part of the process as "the supplement should not present an unreasonable risk of illness or injury, as is assumed in DSHEA." This statement, simple and to the point, requires that to overturn it, data reviewed need to demonstrate that the consumer taking the DSHEA product would, as a result, expose themselves to an "unreasonable risk of illness or injury." The key word is 'data.' The following six chapters in the report, over 120 pages of it, discuss categories of data and how they are to be used. From Human Information and Data (chapter 4), Animal Data (chapter 5), Related Substances (chapter 6), In Vitro Evidence (chapter 7), Interactions (chapter 8), and (chapter 9) importantly, Vulnerable Groups and Prevalence of Use, the report develops how information will be used to produce the safety monograph for a DSHEA product. How one evaluates and

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integrates the available data is critical and addressed in detail. Weighing inconsistent information, proof of harm, and the amount of information needed to draw a conclusion are discussed. The prototype monographs (Chaparral, Glucosamine, Melatonin, Chromium Picolinate, Saw Palmetto, and Shark Cartilage) serve as examples of how to apply the framework.

The final two chapters of the report bear the closest scrutiny. They describe the factors influencing the use of the safety framework and identify the legal and regulatory constraints in place that are in the path of the FDA to discharge its responsibilities to protect consumers. It concludes with recommendations for enhancing the safety evaluation of DSHEA substances. The importance of the latter cannot be underestimated in light of acting FDA Commissioner Crawford's remarks on October 25, 2004, at the annual conference of the Council for Responsible Nutrition, and the Center for Food Safety and Applied Nutrition Director Brackett's testimony before the US House of Representatives' Committee on Government Reform, Subcommittee on Human Rights and Wellness. The former stated that FDA efforts to effect further implementation of DSHEA will first concentrate on "Monitoring and evaluation of product and ingredient safety," while the latter detailed recent FDA and FTC enforcement actions. Both of these statements can be accessed on the FDA Web site www.fda.gov.

Being that, under DSHEA, the manufacturers of dietary supplement products are responsible for assuring that the products they produce and distribute are safe, it is the intent of the ANA through this journal to present for your education a series of editorials dealing with this report and these issues. They will be published throughout this year.

# Managing Cyclical Mastalgia with Absorbable Diindolylmethane: A Randomized, Placebo-controlled Trial

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**ABSTRACT**

This intervention study investigated the efficacy and safety of absorbable diindolylmethane (BioResponse-DIM®) in cyclical mastalgia (recurrent breast pain). Otherwise healthy, pre-menopausal women with cyclical mastalgia were given absorbable diindolylmethane (DIM) or placebo for consecutive three month periods in a randomized, double-blind, crossover study. Breast symptoms were monitored using daily entries on a "breast pain diary" as the assessment tool. Urine and blood samples were collected to confirm safety and the impact of absorbable DIM on estrogen metabolism. Results showed clinical improvement with absorbable DIM. Improvement was not seen with placebo. A statistically significant reduction in duration of breast pain, severity of pain, swelling, and soreness accompanied absorbable DIM use, based on comparison of visual analog pain scores from treatment and placebo periods ($p=.001$-$.03$). In addition, absorbable DIM was shown to increase the ratio of 2-hyroxy to 16-hydroxy estrone metabolites in urine ($p<.05$). Supplementation with absorbable DIM was found to be an effective intervention for cyclical mastalgia. Its use as a dietary supplement deserves further investigation in conditions where modifying estrogen metabolism may be of benefit.

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## INTRODUCTION

Recurrent, monthly breast pain, or cyclical mastalgia (CM), is experienced by 50-70% of pre-menopausal women.[1] Moderate and severe cases of CM result in analgesic use, curtailment of normal activities, and frequent visits to primary practitioners and breast clinics.[2] Despite its frequency, the etiology and optimal management of CM remain uncertain. CM is often inaccurately attributed to the presence of co-existing fibrocystic changes in breast tissue. Studies correlating tissue changes with symptoms show that in about 50% of cases, CM is seen in women with histologically normal breasts.[3] Furthermore, in women with fibrocystic changes who do experience CM, the severity of pain bears no relationship to the associated histologic findings from breast biopsies.[4] The accurate diagnosis of CM requires consistent observation of the onset and worsening of breast pain in the luteal phase of each menstrual cycle, followed by resolution during menstruation. This differentiates CM from other categories of cyst-related and non-cyclical breast pain unrelated to the menstrual cycle. Despite the worsening of CM in association with the luteal

phase when estrogen levels are highest, no connection of CM to disordered production and secretion of ovarian or pituitary hormones has been established. DIM is not known to significantly reduce circulating estradiol or estrone levels. Rather, DIM changes the balance of estrogen metabolites. Studies looking at the association of CM with excess or diminished production of estrogen, progesterone, or prolactin do not explain its occurrence.[5] No prior investigations have reported on the possible relationship of CM to underlying estrogen metabolism.

DIM is the dominant dietary indole from cruciferous vegetables that promotes conversion of estrogen to less estrogenic 2-hydroxy metabolites.[8,9] Indole rich cruciferous vegetables include Brussels sprouts, cabbage, and broccoli. Isolated DIM has been used as a dietary supplement since 1992, and studied in animals for its breast cancer preventive properties since the 1970's.[6] Based on its chemical structure, DIM shares an inducible and overlapping metabolic pathway with estrogen.[7] Due to this overlap, DIM influences estrogen metabolism when absorbed from the diet or dietary supplements. An absorbable formulation of microencapsulated DIM (BioResponse-DIM® [Indolplex®]) has been commercially available since 1998. Absorbable DIM was investigated for CM due to the fact that Danazol, the only approved medication for CM, and alternatively, Tamoxifen (Nolvadex®), both have significant and undesirable side effects associated with routine use.[12] Side effects include hot flushes, nausea, weight gain, and headaches.

Oral use of BioResponse-DIM stimulates 2-hydroxylation of estrogen with amounts of DIM only 2-4 times greater than that obtainable from diet alone.[11,55] Increasing estrogen 2-hydroxylation in peri-menopausal women is desirable, since action of these metabolites has been associated with increased *in vitro* progesterone production[13] and may help to balance the increased estrogen to progesterone activity often associated with early perimenopause.[14] Though other dietary interventions, particularly supplementation with Evening Primrose Oil,[15] have been promoted to relieve CM, fatty acid dietary supplement therapy requires months for a response and provides no benefit for estrogen metabolism. No connection of typical CM to breast cancer has been made. However, a low production of 2-hydroxy estrone relative to 16-hydroxy estrone has now been associated with subsequent breast cancer in prospective and case-control studies in pre-menopausal and post-menopausal women.[16-21] Besides possible cancer protection, 2-hydroxy estrogens result in greater production of methoxyestradiol, a final estrogen metabolite with cardioprotective activity.[22]

## METHODS

### Study Design and Participants

A single-center, randomized, placebo-controlled, crossover study with two consecutive 3-month treatment intervals designed to assess the efficacy of absorbable DIM in CM was performed. The primary endpoints were the duration and severity of breast pain assessed by a detailed "pain diary" completed daily by each subject. Secondly, first morning urine and blood samples were collected at the start of the study, at the crossover point, and at completion. Urine was analyzed to assess the impact of absorbable DIM on estrogen metabolite production, and blood was analyzed to monitor the safety of chronic absorbable DIM supplementation on liver function.

After institutional review and approval of the protocol by the Boulder Community Hospital Institutional Review Board, Boulder, CO, the study was conducted by the Alpine Clinical Research Center, an independent research organization, on contract to BioResponse, LLC, the study sponsor. Following recruitment through newspaper and radio announcements, and upon referral from local physicians,

**Table 1.** Breast Pain Study Enrollment Criteria

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| Recurrent Breast Pain for at least 6 months | **Drugs:** Current Use of Cimetidine or Omprezole |
| History of Breast Pain Before Periods | **Dietary Supplements:** Current Use of Evening Primrose Oil, Borage Oil, Soy Isoflavones, Red Clover Extract, Black Cohosh Extract, or Grape Seed Extract (Resveratrol). |
| Normal Menstruation | Abnormal Menstruation |
| Normal Endocrine Status | Abnormal Endocrine Status, Thyroid Disorders |
| Lack of Suspicious Masses on Breast Exam | Abnormal Breast Exam or Mammogram |

potential subjects were screened to meet inclusion criteria as listed in Table 1.

**Enrollment and Randomization**

Following telephone screening, interested subjects were sent the consent form, and the breast pain diary form to gather prospective data. After a variable period of 1-4 weeks, subjects were seen by a nurse practitioner and, based on a final review of prospective breast pain history, were given informed-consent and enrolled. The intake evaluation included physical examination, body weight measurement, a baseline venous blood sample, and two consecutive first morning urine samples. Instructions were given for subjects to maintain their current diets and exercise routines. Following this, subjects were assigned a patient number and randomized 1:1 to receive either absorbable DIM or placebo. Randomization was accomplished by a third-party-blind supervisor knowing only the patient number and using a computer generated master list. The supervisor had no contact with subjects and provided a three-month supply of either active or matched placebo capsules through the attending nurse practitioner. Upon returning for the 3-month crossover visit, the supervisor was given used capsule containers, confirmed compliance with capsule counts, and issued a second 3 month supply of capsules through the attending nurse practitioner. Each subject's use of absorbable DIM vs. placebo remained concealed from the subject, the evaluating nurse practitioners, and research center personnel. Use of absorbable DIM vs. placebo for all subjects remained concealed until the completion of the study at which time the master list and patient data was released to an independent statistician for analysis. The success of the blinding procedure and secure handling of the master list were monitored and assured by the research center physician director.

**Source and Dose of Absorbable DIM and Placebo.**

Absorbable DIM and placebo capsules were prepared by Tyler Encapsulations (Integrative Therapeutics, Wilsonville, Oregon). Each active capsule contained 60mg of a microencapsulated absorption-enhanced DIM formulation and provided 15 mg of DIM (BioResponse-DIM® [Indolplex®] ), 25% DIM content by formula weight (BioResponse, LLC, Boulder, CO). Microcrystalline cellulose was used as an excipient for encapsulation. Identical placebo capsules contained only microcrystalline cellulose. Absorbable DIM and placebo capsules were each packaged 120 capsules per bottle in opaque, identically labeled bottles. Each bottle was labeled with the study name and instructions to take 4 capsules daily with breakfast (240 mg/day BioResponse-DIM [60 mg/day DIM]). The content of DIM in active capsules was confirmed by independent laboratory analysis for DIM using an established High Performance Liquid Chromatography (HPLC) technique (Alpha Biomedical Laboratories, Petaluma, CA). BioResponse-DIM contains microencapsulated DIM from natural plant sources in a patented, absorption-enhanced delivery system. Data on the absorption and metabolic effects of DIM from BioResponse-DIM have been published.[11,55]

**Plasma and Urinary Metabolite Testing**

As one measure of safety for BioResponse-DIM chronic use, blood plasma was analyzed by standard techniques for a panel of clinical chemistry endpoints of hepatic function. These consisted of albumin, AST (SGOT), alkaline phosphatase (Alk Phos), bilirubin (total, unconjugated, conjugated), and ALT (SGPT). Collected blood was submitted for analysis to be performed at a reference laboratory (Boulder Community Hospital, Boulder, CO). Results obtained from baseline, crossover, and exit visit samples were compared for changes related to absorbable DIM.

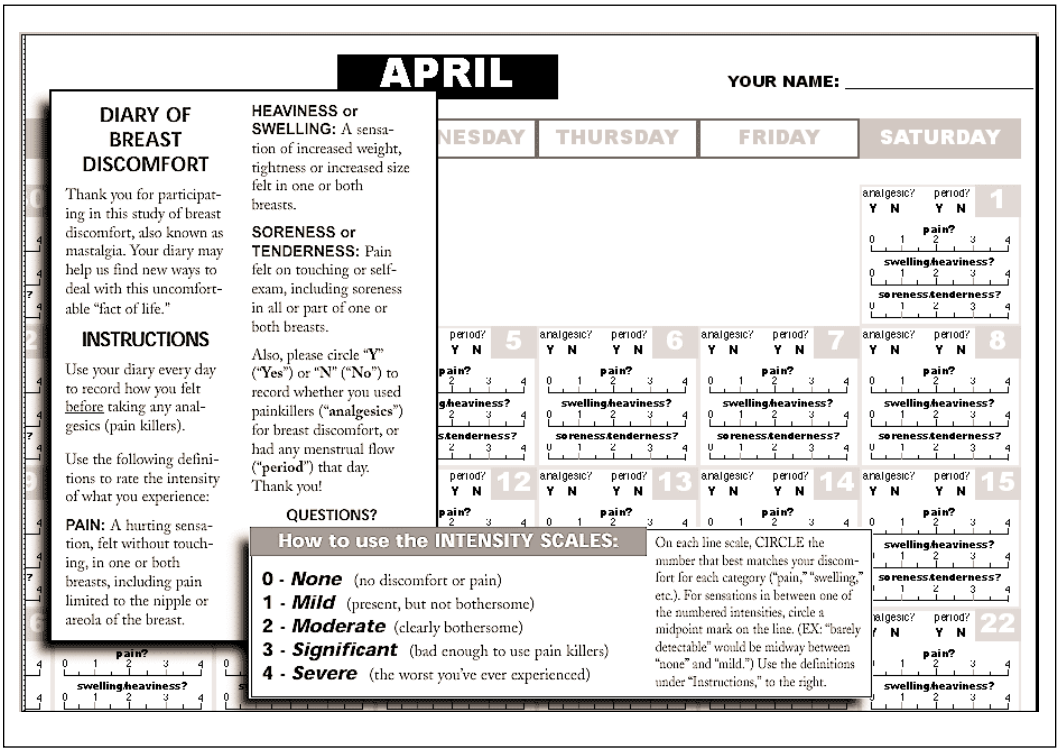
All subjects provided urine samples, (most provided replicate samples on consecutive days) prior to the commencement of the study (baseline), at the crossover point, and then again upon exit. For subjects on the placebo-first protocol, baseline ratios were computed as the average of baseline and crossover samples. First morning urine samples were collected in kits which allowed the immediate freezing of duplicate 30 ml samples after collecting approximately 60 ml samples using collection kits providing 500mg of ascorbic acid per sample as a preservative. The samples were stored at –30°F. Batches of twenty samples were transported frozen prior to being studied for 2-hydroxy estrone and 16-hydroxy estrone levels.. Frozen urine samples obtained at baseline, crossover, and exit points were studied in batches by a collaborating research laboratory for analysis of urinary estrogen metabolites (AMC Cancer Research Center, Lakewood, CO). This testing utilized a commercially available ELISA assay system (Estramet Test, Immunacare Corp., Bethlehem, PA) which determines amounts of 2-hydroxy estrone (2-OHE1) and 16-hydryoxyestrone (16-OHE1) reported in ng/mg of creatinine. This allows calculation of the 2-OHE1/16-OHE1 ratio, which has been shown to have limited intra-subject variation throughout the day and during the menstrual cycle.[23,24] Results are standardized to ng/mg of creatine to account for differences in hydration status and water content of urine. The changes in the 2-OHE1/16-OHE1 ratio were compared statistically.

**Outcome Measures**

Each subject was instructed by her nurse practitioner as to how to enter symptom data on a daily basis into her "pain diary". The Breast Pain Diary was an assessment tool resembling a calendar in which each subject assessed and recorded her breast pain before retiring at night. Each subject rated her degree of pain, swelling/heaviness, and soreness/tenderness for that day according to a linear, visual-analog, scale (See Figure 1.). The Breast Pain Diary employed in the present study was confirmed as an appro-

priate data tool by an independent study of mastalgia which showed that a visual analog scale (VAS) used to assess overall pain, "heaviness", and "tenderness" accurately describes the symptoms of CM.[56] In addition, a "yes" or "no" response was given as to whether menstrual bleeding was present and as to whether analgesics were used that day (see Figure 1).

**Figure 1:** Definitions of breast pain symptoms and pain scale as they appeared on the diary.




Completed diaries were collected and provided to the nurse practitioners for the first three months at the crossover visit, and for the second three months at the exit visit. Phone contact by the nurse practitioner was made during the first two weeks to assist the subjects in determining pain scores and entering data. Raw scores for each parameter were entered into a spreadsheet and analyzed. The documentation of menstruation in association with pain scores was used to assess the cyclical occurrence of pain beginning before menses. The documentation of analgesic use was used to confirm pain severity. Body weight was re-measured at three months and the exit visit.

**Table 2:** Pain intensity and menses (n=no, y=yes) data, as well as calculation of average symptom score for two representative subjects.

| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Average Score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pain | 0 | .5 | 1.5 | 1.5 | 2 | 2.5 | 3 | 3 | 3 | 3 | 3.5 | 4 | 4 | 4 | 0 | 35.5/13=2.73 |
| Menses | n | n | n | n | n | n | n | n | n | n | n | n | n | y | y | |
| Pain | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 2.5 | .5 | 0 | 14/11=1.63 |
| Menses | n | n | n | n | n | n | n | n | n | n | n | n | n | y | y | |

**Statistical Methods for Patient Diaries**

Diary records were evaluated, and a summary score was computed for each of the three symptoms (pain, tenderness (soreness), and heaviness (swelling)) by averaging the positive symptom scores over all days on which symptoms were reported. The duration of symptoms was defined as the total number of consecutive days on which one or more symptoms was present. Table 2 illustrates how these scores were calculated. Each woman reported symptoms for approximately 3 menstrual cycles in each of the two phases of the trial. A repeated-measures general linear model (GLM) was employed to determine if severity and duration of symptoms decreased from month to month while on absorbable DIM.

To test both for placebo effect and for a carryover effect of absorbable DIM, the GLM was employed for both the DIM-first and the placebo-first groups across the three menstrual periods of the placebo phase. This analysis tested for systematic trends across more than two time points while accounting for the dependency in the data induced by a repeated-measures design. Basic descriptive statistics (mean and standard error) were used to compare severity and duration of symptoms for the women during their third period on absorbable DIM and on placebo; differences between these means were compared using paired-sample t-tests. The t-test was used to compute a difference score between scores at the two time points (i.e., crossover and exit) for each individual and then these difference scores were tested for a significant departure from zero.

To provide a more stringent test for the presence of placebo effects, a paired-sample t-test was conducted between the first menstrual period and the third menstrual period symptom scores for each of the symp-

toms (pain, swelling, soreness, and duration of symptoms) for the 15 women who received placebo first. The same statistical technique was used to assess a carryover effect in those patients receiving DIM first followed by placebo.

**Statistical Methods for Urine and Blood Testing**

To determine if absorbable DIM affected the urinary ratio of 2-hydroxy estrone (2-OHE1) and 16-hydryoxyestrone (16-OHE1) as well as the physical symptoms described earlier, a percentage change in 2-OHE1/16-OHE1 ratio from baseline to post-DIM was computed for each subject. This percent change was tested for significant difference from zero using a one-sample t-test. A paired-samples t-test was utilized to determine if 16-OHE1 levels were significantly different from baseline levels following supplementation with absorbable DIM.

Measures of hepatic function (albumin, AST (SGOT), alkaline phosphatase (Alk Phos), bilirubin (total, unconjugated, conjugated), and ALT (SGPT)) were determined for all subjects at baseline, crossover, and upon exit from the study. To test for significant differences among the three levels, a repeated-measures GLM was fit to average baseline, post-placebo, and post-DIM hepatic function values.

## RESULTS

**Patient Population**

During the enrollment period a total of 125 subjects were screened, resulting in 46 women who agreed to enter the study. Of this original sample, 36 subjects provided blood and urine specimens. Of these, 33 subjects provided sufficient breast pain diary data to be included in the final analysis. The remaining 13 of the original 46 subjects were excluded for the following reasons: 2 were judged to be non-cyclical in their mastalgia symptoms, 1 proved to be post-menopausal, 4 withdrew consent due to being "too busy" to complete symptom diaries, 3 were non-compliant with the regimen, 1 did not want to take placebo after an initial response, 1 moved away, and 1 complained of enlarged breasts due to placebo. There were no treatment-related side effects resulting in withdrawal from the study. Of these 33 women, all had sufficient data to contribute to analyses of breast pain, estrogen metabolites and hepatic function data. Four of the 33 women had insufficient diary data to be included in the placebo effect symptom analyses.

**Outcome Results**

No significant changes in body weight were noted comparing body weights at enrollment, the 3 month visit, and exit visit (data not shown).Third menstrual cycle symptoms for all subjects were compared. The paired-samples t-test results demonstrated a statistically significant reduction for breast pain, swelling and soreness during absorbable DIM use. These results are presented in Figures 2a. and 2b. For all subjects during the DIM phase of the study, there was a significant and progressive decrease in symptoms reported from cycle one to cycle three for all measures; all p values were less than .02 (Data not shown). This comparison demonstrated a significant reduction in pain, soreness and swelling while on DIM as well as a carry-over effect of the DIM which caused symptoms to return to pre-DIM lev-

**Figure 2a:** Average third period symptom score for pain, soreness and swelling reported while on absorbable diindolylmethane (DIM) versus placebo.

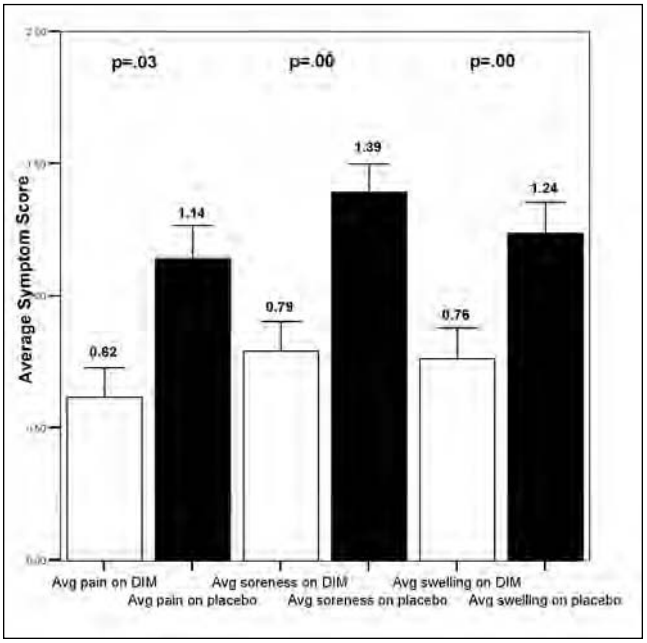
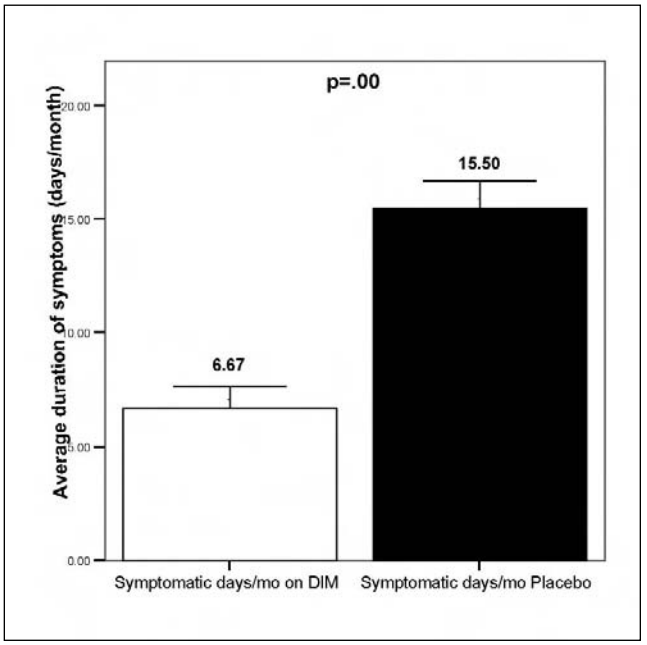



**Figure 2b:** Average third period duration of symptoms reported while on absorbable diindolylmethane (DIM) versus placebo.



els only gradually after cessation of DIM. In the DIM-first group after crossover to placebo, an increase in symptoms was seen across the three menstrual cycles for soreness ($p < .05$) and for duration of symptoms ($p < .01$). An increasing trend was observed for pain and swelling, with p values approximately .11 for each. These findings indicate that the effects of absorbable DIM tend to carry-over for 3 months following cessation of the supplement

For the placebo-first group there were no systematic changes in pain, swelling, soreness, or duration of symptoms from menstrual cycle one to menstrual cycle three; all p values were greater than .4, indicating no evidence for a placebo effect. Results from the t-test comparison of first menstrual period data to second or third period data in placebo first patients also showed no significant placebo effect. Obtained t-values were negative for each measure, indicating an increase in symptom scores from the first to the third period (a reduction in symptoms would provide a positive t-score). The increase in symptom scores during placebo use from the first to the third menstrual period indicates the presence of a placebo effect. However, a statistically important contribution from a placebo effect was ruled out.

**Urine Results**

The mean percentage change in the 2-hydroxy estrone/16-hydroxy estrone ratio (2-OHE1/16-OHE1) due to absorbable DIM showed a 16% increase. This effect was significantly different from baseline ($p = .05$). To determine if there were still carryover effects of DIM on the 2-OHE1/16-OHE1 ratio three months after supplementation had ceased, a percent change in 2-OHE1/16-OHE1 ratio from baseline was calculated for the 15 DIM-first subjects from their sample after three months on placebo. A mean percentage increase of 12% was still present. However, this result was not significantly different from zero for this portion of study group. Figure 3 shows these changes.

The data were subsequently analyzed to determine if there was a significant reduction in the 16-OHE1 level to account for the increase in the 2-OHE1/16-OHE1 ratio from baseline to post- DIM. Paired t-tests were conducted, but no significant differences were present ($p > .10$). Therefore, the increase in 2-OHE1/16-OHE1 ratio was primarily due to an increase in the 2-OHE1 fraction.

**Hepatic Function Testing**

Analysis of hepatic function laboratory values revealed no significant differences among averaged measurements for albumin and Alk Phos. Averaged ALT levels showed statistical but clinically unimportant changes. Post-DIM levels of ALT were 29.5 IU/L as compared to 23.88 IU/L before treatment and 24.26 IU/L post-placebo (all values in the normal range). Also within the normal range, a significant reduction in bilirubin levels was observed following DIM supplementation. Post-DIM levels of conjugated, unconjugated, and total bilirubin were all significantly lower than baseline levels. Means for bilirubin levels and test for differences between baseline and post- DIM are shown in Table 3.

**Figure 3:** (Baseline to 3 months post-DIM).

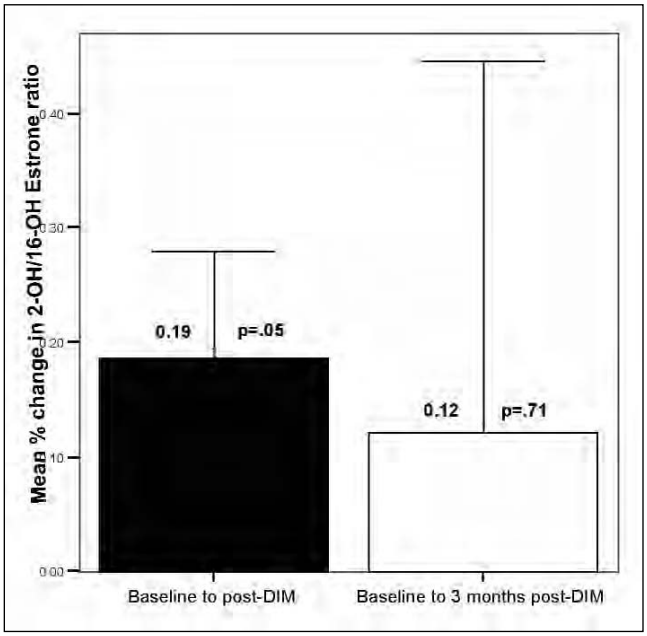



**Table 3:** Mean fractionated bilirubin levels at baseline versus post-DIM (DIM).

| | Baseline | Post-DIM | Statistic |
|---|---|---|---|
| Bilirubin Unconjugated mg/dL | .39 | .26 | t(35)=3.53 p<.01 |
| Bilirubin Conjugated mg/dL | .39 | .32 | t(35)=2.26 p=.03 |
| Bilirubin Total mg/dL | .78 | .58 | t(35)=5.24 p<.01 |

**Table 4.** Individual and averaged results for before and after treatment hepatic function testing.

| Subject | pre-albumin | post-albumin | pre-AST | post-AST | pre-ALK | post-ALK | pre-bili total | post-bili total | pre-bili unconj | post-bili unconj | pre-bili conj | post-bili conj | pre-ALT | post-ALT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4.3 | 3.7 | 22 | 17 | 56 | 60 | 0.7 | 0.6 | 0.3 | 0.3 | 0.4 | 0.3 | 34 | 27 |
| 2 | 4.0 | 4.0 | 16 | 17 | 60 | 57 | 1.5 | 1.3 | 1.2 | 0.8 | 0.3 | 0.5 | 26 | 16 |
| 3 | 4.1 | 4.0 | 20 | 18 | 62 | 56 | 0.6 | 0.6 | 0.2 | 0.1 | 0.4 | 0.5 | 30 | 26 |
| 4 | 3.9 | 4.0 | 22 | 34 | 61 | 64 | 0.7 | 0.6 | 0.3 | 0.2 | 0.4 | 0.4 | 24 | 32 |
| 5 | 3.9 | 3.9 | 17 | 30 | 64 | 65 | 0.9 | 0.7 | 0.5 | 0.3 | 0.4 | 0.4 | 22 | 29 |
| 6 | 3.5 | 3.7 | 20 | 21 | 46 | 42 | 1.5 | 0.9 | 1.1 | 0.3 | 0.4 | 0.6 | 26 | 26 |
| 7 | 3.9 | 4.1 | 23 | 23 | 59 | 56 | 1.4 | 0.9 | 1.0 | 0.5 | 0.4 | 0.4 | 20 | 25 |
| 8 | 3.9 | 4.0 | 16 | 31 | 71 | 80 | 0.7 | 0.5 | 0.4 | 0.1 | 0.3 | 0.4 | 25 | 32 |
| 9 | 4.0 | 3.6 | 18 | 21 | 65 | 75 | 0.5 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 26 | 22 |
| 10 | 3.4 | 3.7 | 18 | 18 | 81 | 92 | 0.4 | 0.4 | 0.0 | 0.0 | 0.4 | 0.4 | 24 | 19 |
| 11 | 3.4 | 3.6 | 26 | 27 | 59 | 64 | 0.7 | 0.6 | 0.2 | 0.2 | 0.5 | 0.4 | 30 | 25 |
| 12 | 3.8 | 3.8 | 23 | 29 | 59 | 73 | 1.0 | 0.5 | 0.5 | 0.1 | 0.5 | 0.4 | 32 | 29 |
| 13 | 4.2 | 4.0 | 10 | 20 | 30 | 55 | 0.4 | 0.8 | 0.3 | 0.4 | 0.1 | 0.4 | 8 | 22 |
| 14 | 3.7 | 3.7 | 18 | 21 | 50 | 58 | 0.8 | 0.8 | 0.4 | 0.4 | 0.4 | 0.4 | 21 | 20 |
| 15 | 3.2 | 3.4 | 25 | 38 | 46 | 43 | 0.8 | 0.7 | 0.2 | 0.3 | 0.6 | 0.4 | 21 | 23 |
| 16 | 3.7 | 3.7 | 20 | 17 | 69 | 65 | 0.7 | 0.7 | 0.2 | 0.3 | 0.5 | 0.4 | 25 | 23 |
| 17 | 3.6 | 3.7 | 18 | 25 | 60 | 68 | 0.9 | 0.7 | 0.3 | 0.3 | 0.6 | 0.4 | 19 | 30 |
| 18 | 4.2 | 3.9 | 26 | 21 | 73 | 66 | 0.8 | 0.5 | 0.2 | 0.0 | 0.6 | 0.5 | 42 | 42 |
| 19 | 4.1 | 3.8 | 29 | 21 | 63 | 73 | 0.7 | 0.4 | 0.0 | 0.0 | 0.7 | 0.4 | 39 | 38 |
| 20 | 3.9 | 4.0 | 20 | 22 | 48 | 54 | 0.8 | 0.6 | 0.2 | 0.3 | 0.6 | 0.3 | 16 | 27 |
| 21 | 3.8 | 3.8 | 22 | 19 | 72 | 67 | 0.8 | 0.4 | 0.3 | 0.1 | 0.5 | 0.3 | 31 | 30 |
| 22 | 3.6 | 3.8 | 18 | 23 | 62 | 65 | 0.2 | 0.4 | 0.1 | 0.1 | 0.1 | 0.3 | 19 | 28 |
| 23 | 4.0 | 3.8 | 25 | 34 | 51 | 48 | 0.8 | 0.6 | 0.5 | 0.2 | 0.3 | 0.4 | 27 | 44 |
| 24 | 3.6 | 3.5 | 20 | 17 | 45 | 39 | 0.7 | 0.5 | 0.4 | 0.2 | 0.3 | 0.3 | 14 | 16 |
| 25 | 3.8 | 3.9 | 22 | 19 | 53 | 57 | 0.6 | 0.5 | 0.3 | 0.1 | 0.3 | 0.4 | 26 | 41 |
| 26 | 3.9 | 3.7 | 20 | 19 | 43 | 43 | 0.8 | 0.6 | 0.4 | 0.3 | 0.4 | 0.3 | 20 | 23 |
| 27 | 3.5 | 3.7 | 16 | 22 | 50 | 52 | 0.7 | 0.6 | 0.4 | 0.4 | 0.3 | 0.2 | 24 | 35 |
| 28 | 3.7 | 3.6 | 20 | 23 | 67 | 56 | 0.4 | 0.5 | 0.0 | 0.2 | 0.4 | 0.3 | 24 | 43 |
| 29 | 3.7 | 4.1 | 23 | 24 | 45 | 53 | 0.8 | 0.5 | 0.3 | 0.2 | 0.5 | 0.3 | 21 | 31 |
| 30 | 3.8 | 3.8 | 23 | 16 | 70 | 79 | 1.2 | 0.8 | 0.8 | 0.4 | 0.4 | 0.4 | 21 | 23 |
| 31 | 4.0 | 3.9 | 19 | 19 | 39 | 48 | 0.5 | 0.6 | 0.3 | 0.6 | 0.2 | 0.0 | 23 | 27 |
| 32 | 4.2 | 4.1 | 16 | 20 | 53 | 44 | 0.8 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 18 | 33 |
| 33 | 4.3 | 4.3 | 19 | 34 | 56 | 53 | 0.6 | 0.3 | 0.2 | 0.2 | 0.4 | 0.1 | 24 | 48 |
| 34 | 4.0 | 3.8 | 24 | 28 | 64 | 61 | 0.5 | 0.2 | 0.3 | 0.1 | 0.2 | 0.1 | 30 | 50 |
| 35 | 3.9 | 3.5 | 24 | 25 | 68 | 62 | 0.5 | 0.1 | 0.2 | 0.0 | 0.3 | 0.1 | 20 | 27 |
| 36 | 4.0 | 3.8 | 20 | 22 | 64 | 57 | 1.4 | 0.8 | 1.2 | 0.8 | 0.2 | 0.0 | 26 | 32 |
| **Mean** | **3.84** | **3.82** | **20.32** | **23.29** | **59.74** | **59.72** | **.67** | **.58** | **.32** | **.26** | **.35** | **.32** | **24.26** | **29.56** |
| (SD) | .25 | .19 | 4.10 | 5.69 | 11.30 | 11.71 | .23 | .22 | .24 | .20 | .15 | .15 | 6.80 | 8.48 |

A summary of before and after treatment hepatic function results for study subjects is presented in Table 4.

## DISSCUSSION

This placebo-controlled trial of absorbable DIM demonstrated a significant reduction in the monthly duration and severity of pain associated with CM. The symptomatic improvement in CM seen with DIM was associated with a measurable increase in 2-hydroxy estrogen metabolite levels in urine samples. Repeated hepatic function testing remained normal in all subjects.

This clinical study supports the use of absorbable DIM as a non-prescription alternative to the pharmacologic use of Danazol and Tamoxifen in CM. There were no significant side-effects attributable to DIM use. This is in comparison to pharmacologic intervention studies utilizing Danazol and Tamoxifen, where significant side effects were reported.[12,25] These studies are compared as to side effects and overall response rates in Table 5. In this comparison, symptom improvement is defined as at least a 50% reduction in an objective measure of breast pain.

Recent, placebo-controlled studies of red clover isoflavones,[57] and chasteberry extract (*Vitex agnus castus*)[58] in CM are also included in Table 5. in order to compare response rates and side effects to those seen in the present study. The red clover study suffered from small sample size and was without statistical significance in the high dose group (n=7, taking 80 mg/day of isoflavones).[57] The study of chasteberry extract showed a comparable overall reduction in the average breast pain score, but required 2-3 months to show this effect.[58] Treatment responses with absorbable DIM were seen within the first month in the present study. One further study using soy protein concentrate and powdered cow's milk as placebo, provided little data for comparison and failed to show a significant reduction in the breast pain score.[59]

Various, prior, uncontrolled intervention studies for CM, including use of the LHRH analogue (Zoladex®),[29] high dose Vitamin A,[30] Vitamin E,[31] and Evening Primrose Oil[15] have also been published. Results from these intervention studies, and anecdotal uses of omega-3 fatty acid supplements and dietary exclusion of caffeine, are difficult to assess or compare because of their lack of placebo-control. In comparison to other controlled and uncontrolled trials, the pain-resolving action of absorbable DIM was clini-

**Table 5.** Comparison of Present Study with Prior, Placebo-Controlled Studies Treating Cyclical Mastalgia (CM).

| Treatment | Duration of Treatment | Subjects Treated N | % of Subjects Reporting Symptom Improvement ≥50% | Side Effect Rate | Reference |
|---|---|---|---|---|---|
| Danazol | 6 cycles | 32 | 72% | 22% | (12) |
| Tamoxifen (Nolvadex®) | 6 cycles | 32 | 65% | 20% | (12) |
| Medroxy-Progesterone (Provera®) | 6 cycles | 18 | 0% | 27% | (26) |
| Bromocriptine (Parlodel®) | 3 cycles | 25 | 65% | 30% mild-moderate 10% severe | (27) |
| Lisuride Maleate (Dopamine Agonist) | 2 cycles | 30 | 56% | 16 % Nausea | (28) |
| Red Clover Isoflavones (Promesnsil®) | 3 cycles | 12 | 30% | 16% | (57) |
| Chasteberry Extract (Mastodynon®) | 3 cycles | 48 | 50% | 8% | (58) |
| DIM (BioResponseDIM®) | 3 cycles | 33 | 42% Pain Reduction 65% ↓ Duration Pain | None | Present Study |

cally important and consistent. In the present study, 25 out of 29 patients with complete pre and post treatment data reported at least a 25% improvement in one or more of the pain parameters while taking DIM. Though averaging of pain scores for statistical analysis made baseline pain scores and improvement seem modest (See Table 2. and Figures 2a. and 2b.), most subjects perceived a distinct and valuable clinical improvement during the study. In contrast to other controlled studies, where a significant placebo effect influenced results, the present study revealed a clinically significant reduction in breast pain without interference from placebo effect. Better control for placebo effect would have been provided by a one month placebo "wash in" for all subjects in the study design. Other deficiencies in the current study include failure to monitor dietary practices with food recall questionnaires during the study. Use of a urinary assay for DIM would also have been desirable to assess compliance. Urinary assay for DIM also helps control for the confounding effect of DIM exposure from increased consumption of cruciferous vegetables during placebo use. A recent clinical study did find DIM in baseline and placebo group urine.[55]

A statistically significant increase in the 2-OHE1/16-OHE1 urinary estrone metabolite ratio indicated that absorbable DIM promoted greater 2-hydroxylation of estrone. For individual subjects however, no correlation of the degree of improvement in breast pain to the increase in the 2-OHE1/16-OHE1 ratio was seen.

Previously, an increase in the 2-OHE1/16-OHE1 urinary ratio following DIM use was shown to require microencapsulated formulation of DIM (BioResponse-DIM®), since use of generic, crystalline DIM failed to increase the ratio.[11] In other prospective studies of pre-menopausal[16] and post-menopausal[17] women, greater 2-hydroxylation of estrogen, using the same 2-OHE1/16-OHE1 assay, has been associated with protection from future breast cancer. In case control studies, a low rate of 2-hydoxylation of estrogen has been linked to breast cancer in women[18-21] and men,[32] uterine cancer,[21] cervical dysplasia,[33] head and neck cancer[34], prostate cancer,[35] and systemic lupus.[36] Regarding estrogen-related risk, 2-hydroxy estrogen metabolites are potentially beneficial.[37] DIM and the 2-hydroxy estrogen metabolites its supplementation promotes are antioxidants.[38,60] 2-hydroxy estrogen metabolites are ultimately converted to 2-methoxy estrogens which demonstrate anti-cancer,[39] anti-mitogenic[40] and vascular-protective effects.[22] Other factors besides cruciferous indoles which increase estrogen 2 hydroxylation include aerobic exercise,[41] low fat diets,[42] and weight-loss.[43]

In the present study, body weights were followed and no changes were noted. Instructions to subjects specified that they maintain the composition of their diets and exercise regimens. No data were collected on details of dietary composition or exercise habits however. When used outside the exercise and dietary constraints of the present study, the metabolic effects of absorbable DIM may add to and complement other behavioral means of increasing estrogen 2-hydroxylation that possibly reduce estrogen-related cancer risk.

Estrogen independent actions of DIM have also been reported. Supplemental DIM prevents chemically induced breast cancer in animals,[6] and controls the growth of induced[44] and transplanted breast cancer in animals.[45] DIM has also been noted to selectively promote programmed cell death (apoptosis) in cancerous but not normal breast epithelial cells.[46-47] DIM is growth inhibitory and apoptosis-promoting for estrogen receptor positive and negative breast cancer cells,[48] and prostate cancer cells.[49] Reported metabolic benefits in DIM-treated breast cancer patients,[55] together with DIM-related *in vivo* uterine[61] and breast protective effects [44,55] support the need for further research into chemopreventive uses of DIM supplements.

The present evidence of effectiveness of DIM in CM, together with the current availability of absorbable DIM as a dietary supplement, encourage further controlled study of DIM's use in CM and other peri-menopausal conditions. These include premenstrual syndrome (PMS), in which anxiety and mood symptoms commonly accompany breast pain and abdominal bloating.[52] In studies of severe PMS, women with the greatest premenstrual elevation of unmetabolized estradiol experience the worst PMS-related anxiety[53] which may be evidence of an underlying, deficient hydroxylation of estrogen.

Absorbable DIM produced no side-effects, no adverse effect on measures of hepatic function, and improved hepatic clearance of bilirubin during the course of this study. This use of BioResponse-DIM at 240 mg/day, which contains 25% DIM by weight,[11] delivered 60 mg/day of DIM. Based on studies documenting the glucosinolate content of Glucobrassicin, the indole precursor of DIM present in cruciferous vegetables,[54] 60-80 mg of DIM can be obtained from about 2.2 pounds of raw cabbage a day.

## CONCLUSION

This placebo-controlled, double-blind trial of absorbable DIM (BioResponse-DIM®) provides preliminary evidence of efficacy and safety in managing CM. Use of absorbable DIM reduced the duration and severity of breast pain in pre-menopausal women with CM. The symptomatic relief observed with DIM was noted after one month of starting supplementation. The relief was statistically significant in all parameters of pain measured after 3 months. Chronic use of absorbable DIM produced no adverse effects on hepatic function, but did result in a significant increase in the urinary 2-hydroxy estrone to 16-hydroxy estrone metabolite ratio. A greater 2-hydroxy to 16- hydroxy estrone ratio has been associated with a diminished future risk of breast cancer.[16,17]

CM is a painful and disrupting condition which often goes untreated due to the lack of side-effect free therapy. CM is experienced by some women in late adolescence, by most women during peri-menopause, and by many post-menopausal women as a side effect of estrogen replacement therapy. Based on the present results, larger controlled studies using absorbable DIM are indicated in CM, in non-cyclical mastalgia, and for pain associated with benign, fibrocystic breast conditions. Absorbable DIM also deserves further study in postmenopausal women as a means of improving estrogen metabolism and reducing breast pain associated with estrogen replacement.

## DISCLOSURE STATEMENT

This study was sponsored by an unrestricted educational grant to the Alpine Clinical Research Center by BioResponse, LLC, a research and development company that produces and markets a formulation of Diindolylmethane (DIM). One of the study authors, Michael A. Zeligs, M.D., is affiliated with BioResponse LLC. As required by the Institutional Review Board, performance of the study and management of clinical data were strictly independent of the study sponsor. At the conclusion of the study, all data were released directly to Dr. Steven Wilson, PhD, an independent University of Colorado based statistician and contributing author.

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# The Use of Fish Oil Supplements in Clinical Practice: A Review

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**ABSTRACT**

Increasing dietary consumption of fish high in omega-3 (n-3) fatty acids is well established as a way to improve numerous health outcomes. The prevention of both primary and secondary cardiovascular events, as well as intervention for such unrelated outcomes as depression and rheumatoid arthritis are now linked with n-3 fatty acid intake. Increasing fish consumption is neither an exact science, nor without risk of consuming toxins of various kinds. The advent of highly purified fish oil supplements, now widely available, has allowed very high levels of n-3 fatty acid consumption for both preventative and therapeutic clinical use. This review will focus on the data concerning fish consumption, fish oil supplements and their fatty acids as it pertains to clinical outcomes, with an emphasis on cardiovascular health.

## BACKGROUND

In the early 1970s, it was observed that high levels of fat intake in the form of long-chain omega-3 fatty acids in Greenland Eskimo populations resulted in fewer cardiovascular events than Western populations who ingested less total dietary fat.[1] In fact, these studies and others prompted the scrutiny of fatty acids based upon whether they were omega-3 (n-3), omega-6 (n-6) or omega-9 (n-9). Fatty acids in the n-3 and n-6 families are considered essential to humans because our metabolism is unable to de-saturate (make a double-bond) between carbons-3 and 4 (n-3) or between carbons 6 and 7 (n-6); counting from the omega or last carbon (See Figure 1 for basic fatty acid information). Typical Western diets provide much in the way of polyunsaturated fatty acids from vegetable sources, which supply high levels of n-6 fatty acids. Data from numerous epidemiological studies have suggested that lowering one's ratio of n-6/n-3 in the range of 3:1 to 6:1 (typical American diet may be as high as 20:1) will have great health benefits.[2] The creation of trans-fatty acids through food processing and cooking further complicates the issues both metabolically and epidemiologically.

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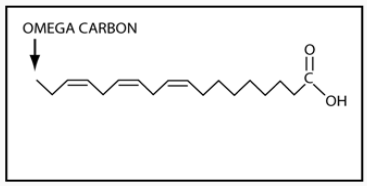
## N-3 FATTY ACIDS

Alpha-linolenic acid (ALA) is an n-3 essential fatty acid found primarily in certain seeds and green leafy vegetables. Flaxseeds are one of the richest sources of ALA. Converting this 18 carbon fatty acid to the 20 and 22 carbon fatty acids found primarily in fish oils requires several steps of elongation and de-saturation (see Fig. 1), reported to be a very inefficient process in adults, suggesting that direct consumption is more reliable.[3,4] And while some data suggests that ALA may help prevent secondary cardiovascular events,[5] most of the focus on n-3 fatty acid research is with consumption of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) from fish and fish oil supplements. In humans, the retroconversion between ingested DHA to plasma EPA seems to be higher than the conversion of EPA to DHA.[107,110]

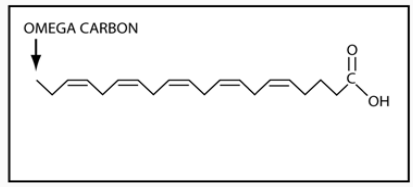
**Figure 1:**

# OMEGA-3 FATTY ACIDS

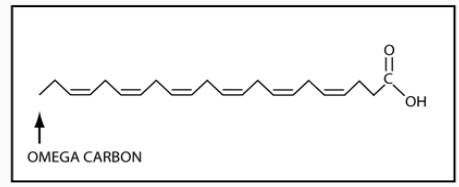
## STRUCTURES




Alpha-Linolenic Acid (ALA) 18:3 n-3



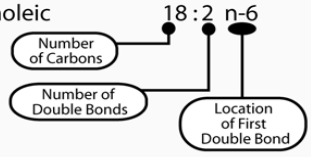

Eicosapentanoic Acid (EPA) 20:5 n-3

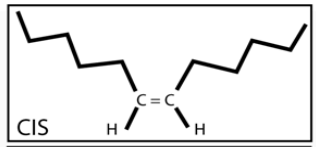


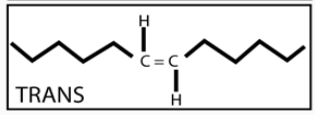

Docosahexaenoic Acid (DHA) 22:6 n-3

## NAMING FATTY ACIDS

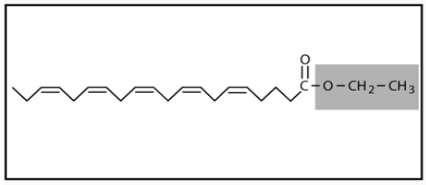
| Common Name | Shorthand |
|---|---|
| Oleic | 18 : 1 n-9 |
| Linoleic | 18 : 2 n-6 |



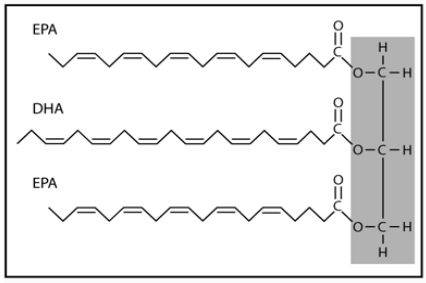




An unsaturated fatty acid double bond is normally in the cis conformation (both hydrogens on the same side). This gives the molecule a bend or kink at each double bond. These bends increase the fluidity or mobility of the fatty acid. When these molecules are heated, exposed to damaging light or oxygen; or partially hydrogenated, trans bonds form. These cause fluid oils to become more rigid (margarine). These new molecules are difficult for the body's enzymes to metabolize. There are significant levels of research looking into the connection between the increased dietary intake of trans-fatty acids and the increased incidence of chronic illnesses.




EPA Ethyl Ester




Triglyceride

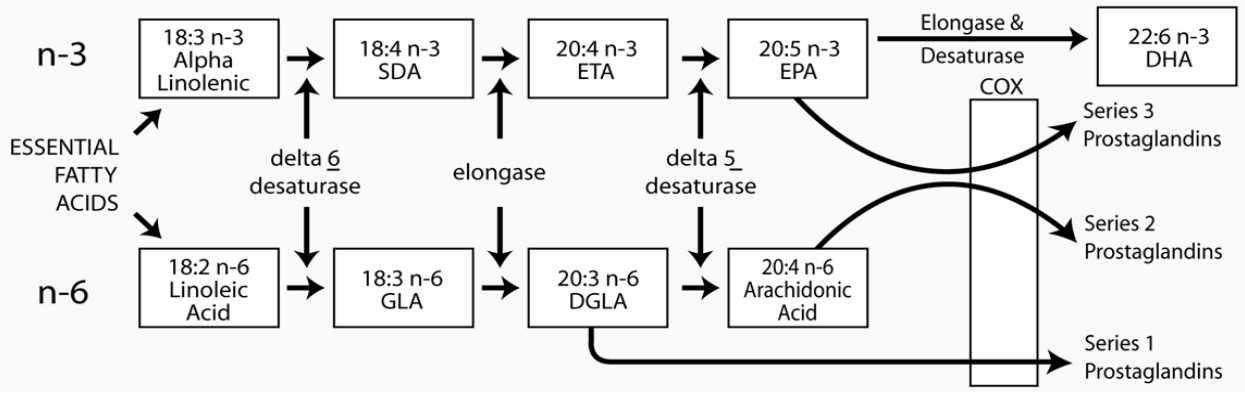
### Typical Fatty Acid Profile of Various Oils & Cooked Fish

( Oils: % of total fat | Fish: mg/3 oz serving )

| | Saturated | | | Mono-unsaturated | | Polyunsaturated | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4:0-14:0 | 16:0 | 18:0 | 18:1 n-9 Oleic | Other MUF A | 18:2 n-6 LA | 18:3 n-3 LNA | 20:5 n-3 EPA | 22:6 n-3 DHA |
| Butter | 22 | 26 | 12 | 25 | | 2.2 | 1.4 | | |
| Olive Oil | - | 11 | 2.2 | 72.5 | | 7.9 | 0.6 | | |
| Corn Oil | - | 10.9 | 1.8 | 24.2 | | 58 | 0.7 | | |
| Canola Oil | - | 4 | 1.8 | 56.1 | | 20.3 | 9.3 | | |
| Coconut Oil | 58.7 | 8.2 | 2.8 | 5.8 | | 1.8 | 0 | | |
| Flax Seed Oil | - | 5.5 | 4.5 | 18 | | 15 | 55 | | |
| Anchovy, canned | 364 | 1127 | 357 | 2501 | 648 | 308 | 14 | 649 | 1098 |
| Catfish, farmed | 95 | 1113 | 271 | 3177 | 338 | 875 | 70 | 42 | 109 |
| Cod | 3 | 72 | 13 | 55 | 34 | 7 | 3 | 88 | 147 |
| Fish sticks, frozen | 59 | 1588 | 1006 | 4241 | 60 | 2327 | 146 | 73 | 109 |
| Haddock | 9 | 96 | 36 | 73 | 55 | 10 | 3 | 65 | 138 |
| Halibut | 61 | 231 | 54 | 394 | 424 | 32 | 71 | 77 | 318 |
| Mackerel | 503 | 1169 | 218 | 1022 | 3935 | 125 | 96 | 428 | 594 |
| Orange Roughy | 6 | 11 | 6 | 305 | 207 | 8 | 2 | 2 | - |
| Salmon, wild | 150 | 689 | 231 | 1427 | 821 | 187 | 321 | 349 | 1215 |
| Salmon, canned | 41 | 1148 | 115 | 908 | 642 | 49 | 49 | 718 | 685 |
| Sardine, canned | 163 | 844 | 292 | 1823 | 1466 | 3012 | 423 | 402 | 433 |
| Trout, wild | 173 | 780 | 225 | 780 | 617 | 245 | 159 | 398 | 442 |
| Tuna, canned | 70 | 503 | 100 | 440 | 225 | 47 | 60 | 198 | 535 |

* Data from USDA Nutrient Data Laboratory website (http:// www.nal.usda.gov/fnic/foodcomp) Accessed January 8th, 2005

## n-3 and n-6 FATTY ACIDS COMPETE FOR ENZYMES DURING METABOLISM




SDA - Stearidonic Acid, ETA - Eicosatetraenoic acid, DGLA - Dihomogammalinolenic Acid, COX - Cyclooxygenase

## CARDIOVASCULAR USES

### Primary Cardiovascular Event Prevention

Numerous reviews have summarized the cardiovascular benefits of fish and fish oil consumption.[6,7,8,9,10] The data concerning primary prevention, however, is less straightforward than the data relating to secondary prevention. In several large cohort studies, the relative risk for CHD and sudden death is reduced with increased fish consumption in men and women,[11,12,13,22,27] while others showed no statistical differences based on fish consumption.[14,15] Plasma EPA and DHA levels measured upon initiation of the Physicians' Health Study did not relate inversely with incidence of myocardial infarction,[16] however in this same group both fish consumption, based on dietary questionnaire, and blood n-3 levels were statistically related to reduced risk of sudden cardiac death.[17,18] In this cohort of 20,551 men, the multivariate relative risk for sudden cardiac death in those consuming 1 fish meal per week was 0.48, compared with men who consumed fish less than once per month.[17] The adjusted relative risk in the 4th quartile of red cell n-3 levels was 0.19.[18]

The Honolulu Heart Program, following Japanese-Americans living in Hawaii, found that the relative risk for CHD mortality was cut in half for heavy smokers (>30 cig/day) if they consumed greater than 2 fish meals per week.[19] Siscovick[20] reported that in a population-based case-control study in King County, WA that both dietary intake of seafood containing n-3 fatty acids and red blood cell membrane n-3 fatty acid concentrations were inversely related to primary cardiac arrest. Both of these associations were dose-related. Among the Nurses' Health Study cohort, fatty fish intake was associated with a reduced risk of thrombotic stroke, while there was no increased risk for hemorrhagic strokes in women.[183] A similar large cohort in the Physicians' Health Follow-up study found the same lowered risk of stroke with fish consumption in men.[184] The American Heart Association recommends that patients without documented coronary heart disease (CHD) eat a variety of (preferably fatty) fish at least twice a week, including oils and foods rich in alpha-linolenic acid (flaxseed, canola and soybean oils; flaxseed and walnuts).[9,21]

### Secondary Cardiovascular Event Prevention

One of the first studies to assess the secondary prevention potential of n-3 fatty acids from fish was the diet and reinfarction trial (DART).[23] The men randomized to receive advice to increase fatty fish consumption (others were advised to increase fiber or reduce fat intake) after recovery from MI, had a 29% reduction in 2-year all cause mortality. Unfortunately, like many lifestyle changes, this advice was difficult to maintain over many decades and both compliance and benefits seem to have been diminished after a decade.[24]

The largest secondary prevention trial to date is the GISSI-prevention trial.[25] In this study, over 11,000 patients (surviving a recent MI) were randomized to receive 1 g/day n-3 fatty acids (capsules containing a minimum of 850 mg EPA and DHA as ethyl esters), 300 mg of vitamin E (acetyl *d,l*-alpha tocopherol), both or placebo. Most of these patients were concomitantly on cardiovascular pharmaceuticals of various kinds, as well as advised about diet and lifestyle changes. Total (RR=0.59) and cardiovascular mortality (RR=0.66) were significantly reduced in the fish oil group as early as 3 and 4 months into the study, respectively. The most dramatic reduction was in sudden deaths, for which relative risks of 0.37 (after 9 months) and 0.55 (42 months) were reported.[26] Among the lipids measured, only triglyceride levels showed significant improvements. In all, there are over 20 randomized, placebo-controlled trials of dietary n-3 fatty acid from fish in CHD patients. A meta-analysis[28] of these trials shows a 3-year average reduction of all cause mortality of 16% and death from MI of 24%. The American Heart Association recommends that patients with documented CHD consume about 1 g of EPA+DHA per day, preferably from fatty fish; EPA+DHA supplements could be considered in consultation with a physician.[9,21]

### Anti-arrhythmic Effects of Fish Oils

Both primary and secondary prevention studies showed that n-3 fatty acid intake was profoundly better at preventing sudden deaths than reducing the incidence of non-fatal MI. Over 50% of the deaths attributed to CHD are sudden deaths (within 1 hour) caused by sustained ventricular arrhythmias. These data suggested that n-3 fatty acids may have anti-arrhythmic effects which initially do not lower the incidence of MI, but prevent many of these events from becoming fatal.[10] This anti-arrhythmic effect has been reported in several animal and cell culture models.[29-33] It is fairly well established that the incorporation of EPA and especially DHA within the plasma membrane of electrically excitable cardiac tissue changes membrane fluidity and modulates the actions of ion channels to prevent the destabilization that permits arrhythmias and ventricular tachycardia.[34-37]

One small pilot study was conducted with 10 patients who had implanted cardioverter defibrillators and repeated episodes of documented, sustained ventricular tachycardia.[38] Compared with baseline, after these patients were infused with n-3 fatty acids, sustained ventricular tachycardia was non-inducible in 5 of the 7 (3 of the 10 patients who ate significantly more dietary fish were non-inducible at baseline). More research, including controlled trials, needs to be done using oral doses of fish oil preparations and clinical outcomes. Leaf et al.[10] recommend that those with a family or personal history of CHD should supplement their diets with 600 mg of EPA plus DHA, and higher, 1 to 2 grams, if there is also a family history of sudden cardiac death.

### Reducing Triglycerides

Elevated triglycerides (TG), both fasting and postprandial, are directly related to the progression of atherosclerosis and are considered independent risk factors for CHD,

especially in women.[39,40] Long-chain n-3 fatty acids from fish like EPA and DHA have shown consistent TG lowering effects in both animals and humans.[42] A meta-analysis of 65 published reports showed TG reduction averaging 25% was typical with fish oil consumption (mean dose 4 g/day EPA + DHA) in both normolipidemic and hypertriacylglycerolemic subjects.[41] These data also show a dose-response relationship between fish oil intake and triglyceride lowering as well as a slight rise in LDL cholesterol (5-10%) and a smaller elevation in HDL cholesterol (1-3%).

Post-prandial (after a fatty meal) plasma TG levels may even be more correlated to atherosclerotic progression than fasting TG levels.[43] Chronic intake of n-3 fatty acids from fish has been shown to reduce post-prandial plasma TG levels.[44] A recent study showed that exercise when combined with fish oils was additive in post-prandial TG lowering.[45] Ten healthy recreationally-active subjects in a cross-over design were tested for changes in fasting and post-prandial (after 1,000 calorie shake- 99% fat after 12-hour fast) TG levels after 5 weeks of fish oil supplementation (4 g/day in 8 capsules of 300 mg EPA and 200 mg DHA each) or exercise (60% $VO_2$max on treadmill for 1 hour), both or neither (control). When exercise was added to fish oil supplementation, the peak plasma TG levels went from 38% reduction (fish oil vs. control) to 50% reduction (fish oil + exercise vs. control). Total area under the TG curve was reduced from 27% to 42% respectively. While both EPA and DHA seem to have triglyceride lowering benefits, DHA may have a more favorable effect. The American Heart Association recommends that under a physician's care, patients who need to lower triglycerides should consume 2 to 4 grams of EPA+DHA per day provided as capsules.[9,21]

**Other Cardiovascular Risk Factors**

In general, fish oil supplements have a favorable, but small effect on HDL cholesterol levels (1-5%). Combined with the more widely observed TG lowering, this (this what?) improves the important TG:HDL ratio. A small study (n=14) was conducted in patients with familial combined hyperlipidemia, noted for their increased cardiovascular risk due to elevated atherogenic lipoproteins and decreased protective lipoproteins.[57] In a cross-over design, patients were given either 4 g/day of a concentrated fish oil preparation in capsules (Omacor- 44%EPA, 36% DHA as ethyl esters) or placebo (corn oil) for eight weeks. As expected, TG levels were lowered significantly (378 to 210), while HDL cholesterol rose a non-statistical 8%. The relative increase in $HDL_2$, a more cardioprotective lipid subfraction, was statistically significant. LDL, but not total cholesterol, was significantly increased in the fish oil group. In one group of hyperlipidemic patients, DHA (4 g/day) had a more significant (29%) increase in $HDL_2$ levels than equivalent levels of EPA.[106] Other clinical trials have also reported that DHA has a slightly more favorable effect on lipid profiles (TG lowering, TG:HDL ratio and lipoprotein fractioning),[107] and post-prandial lipid margination.[108]

It is not uncommon to see elevations in plasma LDL cholesterol after fish oil intake, especially in individuals with elevated triglyceride levels. Since total cholesterol usually remains unchanged in these subjects and it is known that most of the increase is due to an increased shift from VLDL to LDL, the clinical significance of this elevation in plasma LDL cholesterol is not yet known, but LDL subfraction analysis suggests that it is the larger, less-dense (and less atherogenic) LDL fraction which is raised and not the smaller (more atherogenic) LDL particles.[46,186,187] One report suggested a potential down-regulation of LDL receptors to account for part of this phenomenon.[47]

In a group of patients (n=64) with chronic renal failure, assigned to either 2.4 g/day fish oil (4 capsules- 3:2 EPA:DHA) or olive oil for 8 weeks; those receiving fish oil had statistically lower TG (21%), higher HDL cholesterol (8%) and no change in total or LDL cholesterol. A small, non-statistical, drop in Lp(a) was seen in these patients but Lp(a) is very rarely measured in other studies and similar drops were not reported in those studies where it was measured. Also, little effect is reported in lowering high sensitivity C-reactive protein (hsCRP), a marker of inflammation and an independent risk factor for cardiovascular disease.[48,49]

**Metabolic Syndrome and Diabetes**

Metabolic syndrome is a disorder characterized by insulin resistance, high triglycerides, high LDL and low HDL cholesterol, hypertension and central adiposity. An increasingly prevalent condition considered "pre-diabetic," individuals with metabolic syndrome are also at an increased risk of cardiovascular disease even before a diabetes diagnosis.[51,52] In both sucrose and fructose-induced animal models of metabolic syndrome, EPA and DHA from fish oils were able to prevent the onset or diminish several parameters (hypertension, adiposity, dyslipidemias) associated with the syndrome.[53,54] One animal study concluded that insulin-sensitive GLUT4 activity is enhanced in adipocytes (not myocytes) to account for the fish oil's improvement of insulin sensitivity in these animals.[55] While many of the subjects in the TG lowering trials mentioned previously would likely be categorized as having metabolic syndrome, a trial looking at either the prevention or treatment of individuals by this diagnosis as an end-point has apparently not been performed. In one study of overweight treated hypertensive patients (n=69), likely to be deemed as having metabolic syndrome if lipids were reported, combining fatty fish consumption (dietary) with weight-loss had an additive effect on ambulatory blood pressure and decreased heart rate.[56]

Like those with metabolic syndrome, type 2 diabetic patients are characterized with various lipid disorders, insulin resistance and increased risk for CHD. A cohort

within the Nurses' Health study (n=5103) who were free of CHD but with diagnosed type 2 diabetes were evaluated for CHD risk, relative to n-3 intake from fish.[58] After adjusting for age and other cardiovascular risk factors, the RRs for CHD were 0.70 (1 to 3 fish meals per month), 0.65 (2 to 4 times per week) and 0.38 (>5 times per week). Fish consumption in this cohort was more protective against CHD by quintile than it was when looking at all the women in the Nurses' Health Study,[27] implying that n-3 fatty acid supplementation in diabetic patients may prove even more beneficial than in the general population. Consumption of fish is associated with a significantly reduced progression of coronary artery atherosclerosis in women (a higher correlation in diabetic women) with coronary artery disease. Generally, fish and fish oil supplements reduce triglyceride levels and improve HDL levels but seem to have no clinically significant affect on fasting glucose, fasting insulin, $HbA_{1c}$, or glucose tolerance tests in diabetic subjects.[59-61]

In one study, fish *protein* consumption was associated with a significantly lower risk of microalbuminuria in a nested case-control study of 1150 type 1 diabetic patients,[62] although this lowered risk was also reported in a small group (n=16) of type 1 and 2 diabetic patients consuming only concentrated EPA (1.8 g/day).[63] Several animal models have suggested a role for fish oil in general, and DHA specifically, for increasing nerve conduction velocity in diabetic neuropathy. Collectively, these data suggest that diabetic patients should consume 1 to 2 grams per day of n-3 fatty acid from fish, balanced between EPA and DHA.

**Hypertension**

There is a dose-dependent inverse relationship between n-3 fatty acid intake and blood pressure in hypertensive patients, but little effect is noted in normotensive or borderline hypertensives. A meta-analysis of 31 placebo-controlled trials found an average -0.66/-0.35 mm Hg drop in systolic/diastolic blood pressure per gram of n-3 fatty acid consumed in hypertensive patients.[67] Many of these trials used doses in excess of 5 grams per day and were associated with gastrointestinal complaints. Another meta-analysis reported an average reduction of 5.5/3.5 mm Hg in hypertensive patients given at least 3 g/day of n-3 fatty acids. Fish oil consumption (~3.6 g/day from diet) had an additive effect when combined with weight loss in overweight hypertensives (-6.0/-3.0 fish alone, -5.5/-2.2 weight loss alone, -13.0/-9.3 mm Hg combined).[56] The authors conclude that given the magnitude of the BP reduction with the fish/weight loss combination, withdrawal of antihypertensive therapy may have been possible.

DHA and EPA have been tested separately for their hypertensive activities. Mori et al. has reported that 4 g/day of DHA, but not EPA, reduces ambulatory blood pressure and has favorable effects on arterial compliance.[104,105]

**Additional Cardiovascular Mechanisms**[77]

Discussing the various potential biological mechanisms in detail is beyond the scope of this review. For the sake of those interested in pursuing this avenue, however, a list of reported potential mechanisms attributed to n-3 fatty acids and several references are included below.

- Anti-inflammatory[68-73]
- Arterial compliance[74,75,76]
- NO- induced endothelial relaxation[78,79]
- Reduced asymmetric dimethyl arginine (ADMA)[80,81]
- Reducing atherogenic adhesion molecules[82,83,84]
- Anti-thrombogenic[85,86,87]
- Stabilizing atherosclerotic plaques[88]
- Peroxisome proliferator-activated receptors (PPAR) regulation[89,90]

## NON-CARDIOVASCULAR USES

**Anti-inflammatory- Rheumatic Diseases**[115,116]

The well-known pathways which covert the 20 carbon n-6 fatty acid arachidonic acid into pro-inflammatory cytokines is often termed the arachidonic acid cascade. Key enzymes in the formation of pro-inflammatory prostaglandins and leukotrienes are the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Inhibition of these enzymes is one of the most popular anti-inflammatory mechanisms in the pharmaceutical trade. Since the substrate for each of these enzymes is a 20 carbon fatty acid, eicosapentanoic acid (EPA) is capable of both competing for the use of the enzyme as well as forming eicosanoids which function to counteract the activity of eicosanoids derived from arachidonic acid.[127] These mechanisms have led to the proposal that increasing n-3 (especially EPA from fish) and lowering n-6 fatty acid intake would have a favorable benefit on the overall inflammatory burden, particularly in individuals with chronic conditions such as rheumatoid arthritis.[117,118]

Omega-3 fatty acids from fish oil have been studied extensively in patients with rheumatoid arthritis.[119] Meta-analysis data suggest a modest improvement in tender joints and morning stiffness with the addition of fish oil supplementation.[120] Dosing and fish oil content vary widely in different clinical trials. The most significant benefits seem to require at least 3 grams/day, although benefits were seen in some trials with 2.6 grams/day,[121] 30 mg/kg/day[122] and 40 mg/kg/day.[123] Significantly more benefit is seen when patients who use fish oil supplements are also consuming a low arachidonic acid, anti-inflammatory diet.[122]

The role of fish oils has also been explored in patients with inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Reviews of the various clinical trials have shown that doses as high as 4.5 and 5.4 grams per day have limited benefit on preventing relapses, but often

reduce the dependence on steroid therapy and dramatically reduce inflammatory markers.[124] A specially prepared enteric-coated, free fatty acid preparation (1.8 g/day EPA, 0.9 g/day DHA) was able to significantly reduce the level of relapse compared to placebo in a group of Crohn's disease patients (n=78).[125] Another group recently reported that stimulated T-cells and monocytes taken from Crohn's disease patients supplemented with fish oil (1.6 g/day EPA, 1.08 g/day DHA- non-enteric coated) and an antioxidant blend (Vit. A, C, E, selenium, manganese ) produced lower interferon-gamma and $PGE_2$, compared to placebo.[126] In general, these data suggest that individuals with inflammatory bowel conditions may be benefited by increasing fish oil intake equivalent to 2.5-5 grams per day.

**Depression and Other Mood Disorders**

Long chain n-3 fatty acids are important components of membranes within neurological organs and tissues. They affect membrane fluidity and excitability, influence synaptic function, and perhaps serotonin and dopamine metabolism.[128,145] In several epidemiological studies, fish consumption is related to decreased risk of depression, especially in women.[129,130,131] Although not all cohort studies proved statistically significant,[132,133] a recent case-controlled study (China) reported that low red blood cell EPA levels are associated with increased risk for attempting suicide.[139] Previous reports suggest there is a link between violent suicides and seasonal intake of EPA.[140]

Several clinical trials have used n-3 fatty acids to treat depression and related disorders.[141] Most of the studies to date have used a preparation of pure EPA (EE form). Peet et al.[142] reported that 1 gram (but not 2 grams) of EPA improved depression scores in patients (n=17 each group) with ongoing medicated depression. However, Nemets et al.[143] reported that similar patients (n=20) receiving 2 grams per day of a comparable preparation had highly significant reduction in Hamilton depression scale scores (mean 12.4 point reduction vs. 1.6 for placebo). This same group attempted to use this preparation at the same dose to treat medicated patients with obsessive compulsive disorder (OCD) without success.[144] Pure DHA (2 g/day) had only a small, non-statistical benefit in patients with major depression. Bipolar patients given high doses of fish oil (6.2 g EPA/3.2 g DHA) had a significantly longer period before relapse than similar patients taking olive oil.[147] Physicians treating patients with depression or related disorders should consider measuring patient serum fatty acid levels and including fish oil supplements (particularly EPA) at 1-2 grams per day.

**Maternal and Infant Care**

Maternal fatty acid levels, especially DHA levels steadily drop in late pregnancy,[135] increasing risk for post-partum depression.[136,137] A meta-analysis of 41 studies showed that lower fish consumption and breast milk DHA content were associated with increased risk for post-partum depression.[134] Low doses of DHA (200 mg/day -algae-derived) given post-delivery, however, were unable to significantly lower symptoms of post-partum depression.[138]

The role of n-3 fatty acids in maternal gestation and parturition, as well as offspring development has been reviewed elsewhere.[148] Generally, women with higher n-6 to n-3 intake have a higher likelihood to deliver prematurely. This phenomenon is thought to be related to changes in eicosanoid production (prostaglandins, leukotrienes) which take place prior to parturition. Epidemiological studies suggest that gestation is generally longer in women with higher intake of n-3 fatty acids from fish in some cohorts,[149,150] but not in others.[151,152,153] High n-6 to n-3 fatty ratios also correlate to an increased risk for preeclampsia.[154,155] Intervention trials, during high risk pregnancies have shown some improvement in prolonging gestation (2.7 g/day n-3),[156,157] but not in pregnancy related hypertension.[157-160]

Rapid growth in the brain occurs during the last trimester of pregnancy and the first several postnatal months. The need for maternal DHA is critical during these months since fetal and newborn fatty acid metabolism is inadequate to provide proper levels of DHA for brain development. Several reports suggest that maternal supplementation of fish oils or DHA alone during the third trimester and while breast-feeding can improve cognitive development in newborns,[161] improve sleep patterns (a measure of brain development),[162] and even increase IQ scores at age 4.[163] Maternal fish oil supplementation (3.7 g/day n-3, 56% DHA) in atopic women (offspring considered at high risk for allergic diseases) significantly increased breast milk levels of the protective Immunoglobulin A (IgA) and CD14.[164] Children born from these mothers have reduced levels of allergic related cytokines and allergen-specific immune responses.[165,166,167,168] Children at high risk for atopic diseases had reduced allergy-related cough at age 3 if they were supplemented with fish oil (500 mg of tuna oil/d- 185 mg n-3) from 6 months to 3 years.[169] Eating high levels of n-3 fatty acids directly from fish is contraindicated in young children and pregnant women due to the potential for ingesting mercury and other toxins. Fish oil supplements, virtually free of these toxins,[170,171] are safer and allow for specific dosing regimens. Many liquid as well as capsule preparations can be used which provide varying levels of DHA, some of which are specially prepared and flavored for children.

**Ocular and Cognitive Health**

As a specialized portion of the nervous system, the retina has one of the highest levels of long-chain fatty acids in the human body; especially concentrated is the level of DHA.[172,173] Infant visual acuity is diminished in n-3 deficiency. Children supplemented with DHA (115 mg/day) from 6 months to 1 year of age had significantly better improved visual acuity than similar control children.[174] The long-term visual benefits for infant supplementation is not

yet known. In adults, fish and DHA intake (determined by food questionnaire) reduces the risk for age-related macular degeneration.[175,176] Preventative or intervention trials in patients with or at risk for macular degeneration have not been published.

The relationship between DHA and retinitis pigmentosa (RP) is currently being investigated. RP patients have lower levels of DHA,[177] partly due to reduced activity of the enzyme delta-5-desaturase.[178] Despite this relationship, trials attempting to slow the progression of RP with supplementation of DHA have been unsuccessful,[179,180] although chronic vitamin A users who added 1200 mg/d of DHA had some slowing in progression after 2 years.[181]

Increased dietary intake of fish and DHA (but not EPA) is correlated (cohort of 815) with a decreased risk of Alzheimer's disease.[182] Whether this correlation will prove to be of preventative or therapeutic benefit is yet to be determined. Studies also suggest that DHA is protective against dendritic cell damage in a mouse model of Alzheimer's disease.[185]

**Fish Oil- The Product**

Recommendations to increase fish consumption are not always straightforward. Some fish have high levels of EPA and DHA; others do not (see chart figure 1). How the fish is prepared also has a significant affect on whether these long-chain fatty acids will be beneficial. In a population-based cohort study, dietary fish consumption was correlated with increased plasma n-3 levels and reduced risk of cardiovascular death in individuals consuming tuna or other similar fish (broiled or baked), but neither was associated with fried fish or fish sandwiches (fish burgers).[91] The same group reported similar differences for reducing the risk of atrial fibrillations in these different populations based on type of fish consumed.[92] The susceptibility to loss or modification of EPA and DHA has been reported in various cooking processes, especially deep-frying.[93] The additional potential hazard of consuming environmental toxins such as methyl mercury and other heavy metals or pesticides like DDT, DDE or PCBs is a concern for many. The Environmental Protection Agency warns those most at risk (pregnant women, nursing mothers and their infants and young children) to limit fish intake to avoid potentially dangerous mercury levels.[94] Several advantages of fish oil supplements directly address these concerns. Levels of EPA and DHA are consistently dosed in capsule or liquid products. Levels of heavy metals and pesticides can be dramatically reduced, often below detectible limits, when using fish oil supplements in lieu of consuming more fish.[170,171] Fish oil is inherently more susceptible to oxidation, requiring that most products contain additional fat-soluble antioxidants such as natural vitamin E, fat soluble ascorbates or other natural antioxidants to protect them from becoming rancid under normal storage conditions.

Commercial fish oil is a by-product of the fish meal industry. It is typically a blend of many different fish species including mackerel, anchovies, sardines, tuna, salmon and others. The raw oil from these fish is then purified and concentrated by removing (hydrolyzing) the individual fatty acids from the fish triglycerides so the various fatty acids can be separated and concentrated. This process allows for the separation of contaminant toxins, proteins (which may increase allergenicity and burping), and other non n-3 fatty acids. These concentrated fatty acids remain as free fatty acids (FFA) before they are stabilized by esterification to ethanol (ethyl esters, EE) or further esterified back to a glycerol backbone to create a re-esterified triglyceride (rTG). Both EE and rTG forms of varying concentration (30-70% EPA+DHA) are used in the dietary supplement industry throughout the United States.

Few studies have looked at differences between fish oil supplements provided as EE or rTG. One study reported that plasma EPA and DHA levels were higher when equivalent levels of these fatty acids were consumed directly from salmon than from fish oil supplements provided as ethyl esters.[95] Whether the ethyl ester form diminished or some fish component enhanced bioavailability is not known. Several studies have shown that plasma bioavailability of the EE form is less than 50% of that from the rTG form.[96,97,98] Other studies, however, show no difference in bioavailability between these two forms.[99,100] All of these studies were uncontrolled and involved very few subjects. Dyerberg et al.[114] completed a study involving 72 subjects, comparing the bioavailability of EPA and DHA from natural fish triglycerides, EE, rTG, cod liver oil and FFA. They found that compared to natural fish TG (100% standard), the bioavailability of EPA and DHA combined was highest from the re-esterified TG (124%) and lowest from EE (73%). The EPA and DHA incorporated into phospholipids was 62% and 290% greater when consumed as rTG rather than EE. At this time there are no trials comparing the potential differences in the EE and rTG forms as it pertains to clinical outcomes (triacylglyeride lowering, hypertension, etc.); many reports don't specify the forms used. Since data suggests that individuals are likely to absorb the rTG form better, and lipase and biological incorporation of the EE is diminished,[99,101] clinical trials should be done to assess whether the rTG form may have better clinical outcomes, or require lower doses for equivalent results. Consistent results at a lower dose would help increase compliance and reduce both side-effects and cost.

As with any dietary supplement, choosing a high-quality fish oil product is important. The Council for Responsible Nutrition (CRN), along with many of the leading fish oil manufacturers in the world, published a monograph in 2002 outlining various quality aspects which the industry should use to regulate fish oil products.[102] This monograph stipulates upper limits for mercury and other

heavy metals, pesticide levels and oxidation levels such as peroxide and anisidine values. These guidelines have been adopted by the United States Pharmacopoeia (USP) for their current n-3 fatty acid from fish oil monograph.[103] Additionally, some companies monitor the production from catch to finished product in order to provide kosher products to the market.

**Side-effects and Contraindications**

High-dose fish oil supplementation is extremely well tolerated in nearly all individuals. The most common side-effect is a fishy aftertaste or "burping" associated with high doses. When products are consumed with meals and carbonated beverages are avoided, this unpleasant feature is dramatically reduced. The complete purification of the fatty acids from fish proteins virtually eliminates the potential for allergic components in the fish oil supplements. Because fish oil is prone to oxidation, consuming high doses without additional antioxidant protection (from diet or supplemental sources) may increase vulnerability to lipid peroxidation, especially in warm and sunny climates. While the in vivo consequences of this vulnerability are still being debated, antioxidant supplementation should be recommended for every individual consuming high amounts (3 grams or more) of fish oil daily. These high doses can be consumed directly from bottles for those wanting to avoid gelatin capsules due to concerns about consuming non-fish animals in general or bovine-derived products specifically.

The most frequent contraindication concern is the combination of high dose fish oil with pharmaceutical drugs that affect blood clotting (coumadin, aspirin, etc.), used by many cardiovascular patients. A group of 250 patients who had undergone coronary artery bypass surgery were given 4 g/day of fish oil concentrate and either aspirin (300 mg/d) or warfarin therapy.[111] Compared to patients not receiving fish oil, these patients had no increase in bleeding time. Another report showed no change in INR when 6 g/day of fish oil was given to patients on chronic warfarin therapy.[112] However, one case report has been published of a woman (67 years old on coumadin, 1.5 years at 1.5 mg/day) who had an increased INR (2.8 to 4.3) in the month she doubled her fish oil supplement from 1 to 2 g/day.[113] These data suggest that the concern for bleeding times is generally not an issue, but INR should be checked in patients on both warfarin and fish oil therapies.

## CONCLUSION

Epidemiological evidence is quite clear in demonstrating numerous health benefits in consuming long-chain polyunsaturated n-3 fatty acid from fish, especially as a ratio to n-6 fatty acids derived from vegetable oils. Even an "Omega-3 Index" of RBC EPA and DHA levels is being proposed as a routine laboratory test for measuring cardiovascular risk.[109] In the past decade, the clinical use of fish oil supplements has greatly increased, as has the data supporting their use. While dietary and lifestyle changes are ideal ways to modify a number of cardiovascular risk factors, many individuals with personal or family history of cardiovascular disease cannot safely consume high levels of n-3 fatty acids from fish alone, or do not maintain the dietary habit.[24] Since fish oil supplements have been shown to have beneficial effects on nearly every risk factor for cardiovascular disease, and so many individuals are currently at risk, the recommendation to use these supplements in clinical practice is encouraged. There are few patients who would not realize some benefit by increasing their fatty fish consumption or adding fish oil supplements to their daily routine. Patients with previous CHD, hypertriglyceridemia, hypertension, type II diabetes or metabolic syndrome should be taking at least 2 g/day of n-3 fatty acids from fish oil daily via supplements. Pregnant women should be encouraged to consume fish oil supplements to increase n-3 fatty acids, particularly DHA throughout the second half of pregnancy and while breast-feeding.

**Disclosure statement:** This author is affiliated with a company, Ortho Molecular Products, that manufactures and distributes dietary supplements, including fish oil products.

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# Appropriate Spectrum Vitamin E and New Perspectives on Desmethyl Tocopherols and Tocotrienols

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**ABSTRACT**

Research in the past decade shows that the commonly used non-desmethyl vitamin Es (e.g., alpha-tocopherol and alpha-tocotrienol) do not share the beneficial effects of desmethyl vitamin Es (e.g., gamma and delta isomers of tocopherols and tocotrienols). Research also shows that high levels of alpha-tocopherol may attenuate the bioavailability and functional activity of other vitamin E isomers. In general, desmethyl tocotrienols are much more bioactive than desmethyl tocopherols, especially in cancer inhibition.

This paper delineates the role of desmethyl tocopherols and desmethyl tocotrienols in biological studies and in human health. A new perspective is presented for applications of delta-tocotrienol, gamma-tocotrienol, delta-tocopherol and gamma-tocopherol that are consistent with the emerging science of vitamin E. The paper concludes that formulated vitamin E should be "appropriate spectrum" and not merely "full spectrum" based on 35-40 mg of daily consumption (DC) of vitamin E in foods. Formulated "appropriate spectrum" vitamin E should more closely reflect the composition of our diet, and is therefore well suited for maintenance; (1X DC); prevention (10X DC) and treatment (50-200 mg/day desmethyl tocotrienols) formulations may require higher doses.

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Diversity brings out the best in unique cultures. This diversity can be appreciated not only in human societies but also in the plant kingdom. For example, there are in excess of 600 naturally occurring carotenoids in plants yet only a handful, namely beta-carotene, lycopene and lutein, are actively being researched. Similarly, there are more than twelve vitamin Es found in nature but only alpha-tocopherol is primarily being studied. This paper calls into question the suitability of unqualified use of large doses of alpha-tocopherol as well as the unqualified use of the "full spectrum" vitamin E. The advent of "appropriate spectrum" vitamin E for human health is a derivative concept[1] from punctuated research development. This review addresses recent research developments to delineate the functional roles of desmethyl tocotrienols and desmethyl tocopherols apart from common alpha-tocopherol.

## VITAMIN E IN PLANTS AND FOOD

Dicotyledoneous plants (e.g., soy, peanut) typically contain tocopherols, predominantly as gamma-tocopherol, and secondarily as delta-tocopherol and alpha-tocopherol. Monocotyledoneous plants (e.g., palm, rice) typically contain tocotrienols, predominantly as gamma-tocotrienol, and secondarily as delta-tocotrienol and alpha-tocotrienol. Beta-tocopherol and beta-tocotrienol are almost insignificant in abundance in any plant and negligible or unknown in activity. Dicotyledoneous plants that contain tocopherols may contain lesser (~5%) tocotrienols, and monocotyledoneous plants that contain tocotrienols may contain more (~30%) tocopherols.[2] Tocopherol-free tocotrienols are rare and found only in few plants, including annatto.

Much information is available on composition of tocopherols, especially alpha-tocopherol, in both whole and processed foods. These published works do not typically include tocotrienol composition, even though tocotrienols and tocopherols are often found together. Tocotrienols exist naturally in oils and fats, and in whole foods as well as processed foods. Table 1 summarizes the sources of dietary tocotrienols in the American diet.

Tocotrienols may also be found in prepared foods (e.g., macaroni, brown bread, Danishes, doughnuts, rolls, cake mixes, most breakfast cereals, baby formulas) and snacks (e.g., candy bars, cookies, biscuits, crackers, popcorn, and potato chips).

## CHEMISTRY AND HISTORY OF VITAMIN E

The 6-hydroxychroman moiety with a lipid-soluble side chain (either a longer *phytyl* for tocopherol or a shorter *farnesyl* for tocotrienol) constitutes the collective term now known as vitamin E (Figure 1). It is generally believed that there are only four tocopherols and four tocotrienols in nature. However, there are at least 12 known E vitamins, including two new tocopherols and two new tocotrienols, and this number is likely to increase in the future. Historically, the first vitamin E (alpha-tocopherol) was discovered as a vital nutrient which offers protection against fetal resorption.[3] A burst of research activities followed after the initial discovery of vitamin E by Herbert Evans of the University of California Berkeley: its isolation from plants,[4] chemical identification,[5,6] complete synthesis,[7] and antioxidant activity[8] were all first reported before 1940. Other tocopherol discoveries soon followed,[9,10] while the tocotrienol discoveries appeared much later.[11,12] Even though reported in publications, these newly discovered tocopherols and tocotrienols were less known and even less understood than alpha-tocopherol. Interestingly, tocotrienols were erroneously named tocopherols, an error that remained uncorrected for many years.[13] It was actually not until recently that this correction was made.[14] Tocotrienols' ability to lower lipids was first reported in the early 1980s, and in the 1990s they were implicated for inhibition of cancers and reduction of cardiovascular diseases.[15] The 1990s saw the scientific understanding of gamma-tocopherol, and to a lesser extent, delta-tocopherol. This same period also saw the delineated functions of gamma-tocotrienol and delta-tocotrienol.

Commercialization of natural soy-derived tocopherols occurred in the 1950s. The eminence of alpha-tocopherol prompted many companies to chemically convert soy and corn tocopherols (with crop abundance typically less than 20% alpha-tocopherol) to 100% alpha-tocopherol. The "natural" alpha-tocopherol on the market is, in fact, synthetic. These soy- and corn-based tocopherols are synthesized to (RRR- or d-) alpha-tocopherol via the addition of one methyl group to gamma-tocopherol and two methyl groups to delta-tocopherol. This C5 methyl addition is the primary chemical process.

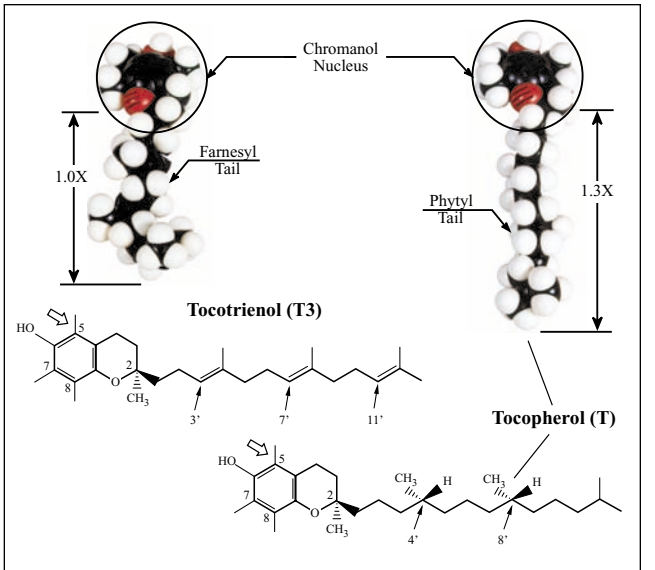
There was yet another motivating reason for this chemical conversion to alpha-tocopherol. The International Unit (IU) is used as a unit of measurement for the recommended daily allowance (RDA) for vitamin E and the RDA value

**Table 1.** Tocotrienols in the American Diet*

| | |
|---|---|
| Oils and Fats | Wheat bran, wheat germ, corn, rice bran, palm, oat, oat bran, grapeseed, coconut, barley, margarine, lard |
| Vegetables and Grains | Carrots, wheat, barley, corn, oat, rice, rye, cauliflower, broccoli, peas |
| Fruits | Avocados, apricots, blueberries, black currants, grapes, olives |
| Nuts and Seeds | Almonds, cashew, coconut, macadamia, pistachios, annatto |
| Meats and Eggs | Chicken, liver, pork, veal, egg |

*Summarized from Eitenmiller and Lee (2004) and Sheppard, A. et al. (1993)

**Figure 1:** Molecular and Chemical Structures of Vitamin E




| Methyl Group Position(s) | Tocotrienol* | Tocopherol* |
|---|---|---|
| 5,7,8 - Trimethyl | alpha-T3 (*58864-81-6*) | alpha-T (*59-02-9*) |
| 5,8 - Dimethyl | beta-T3 (*490-23-3*) | beta-T (*16698-35-4*) |
| 7,8 - Dimethyl | gamma-T3 (*14101-61-2*) | gamma-T (*54-28-4*) |
| 8 - Monomethyl | delta-T3 (*25612-59-3*) | delta-T (*119-13-1*) |

*CAS number in parenthesis

of 30 IU was determined based on the vitamin's capacity to prevent *hemolysis* of the red blood cells.[16] The popular use of 'IU' replacing the 'mg' in then newly established RDA was welcomed by the industry because alpha-tocopherol has the highest IU/mg value as compared to all other vitamin Es. However, the established 30 IU did not relate to Evan's initial discovery that first named alpha-tocopherol as a "vitamin" for protection against fetal resorption. To further confound the issue, the United States Pharmacopeia, unlike the RDA, defines an IU of vitamin E as 1 mg of all rac alpha-tocopherol acetate based on the rat *fetal resorption* assay.[17] Nonetheless, the established RDA catapulted the use of alpha-tocopherol (1.5 IU/mg), and put the two major nature-derived tocopherols — gamma (0.15 IU/mg) and delta (0.05 IU/mg) — at a distinct disadvantage. This 10 to 30 fold IU/mg difference clearly prompted commercialization of the chemically converted "natural" alpha-tocopherol. These IU factors are largely overlooked because of the repeated discussions that focus on natural-versus-synthetic alpha-tocopherol, where the IU/mg value is 36 to 100% higher for the natural form.[18,19]

## ANTIOXIDANT AND LARGE DOSES

Much has been reported about the well known antioxidant properties of tocopherols and tocotrienols. For example, alpha-tocotrienol is a 40 to 60 times more potent antioxidant for protecting rat liver lipids than alpha-tocopherol.[20] This protection was attributable to the efficient membrane mobility of alpha-tocotrienol[21,22,] possibly because of its shorter farnesyl side chain that provides less anchoring (Figure 1). However, conflicting results have appeared in the literature as to the relative antioxidative properties of vitamin E isomers. The biological milieus for which such studies were conducted are complex and diverse so that cross comparison between studies is not meaningful. When studies are conducted in the same system, tocotrienols are equal to or greater than tocopherols as antioxidants.[20-24] Still, it is certain that all vitamin E isomers are potent lipid antioxidants. Readers are directed to recent reviews on the comparative potential of tocopherols and tocotrienols as oxidative protectants.[21,22,25]

The discovery of alpha-tocopherol as the first vitamin E, and its unique antioxidant properties and prevalence in the human body, has led scientists to discount other vitamin E compounds to near obscurity. The escalating amount of supplemental daily use from 1X RDA (30 IU) to about 70X RDA (>2000 IU) is problematic, and seems to lack rationality in science except for the perceived notion that "more is better" and "alpha-tocopherol is *the* vitamin E". The null effects of numerous alpha-tocopherol trials could have signaled the lack of benefits from large doses earlier.[26] The recent Johns Hopkins meta-analysis[27] appears to slam the brakes at 400 IU per day as the safe level. This recommendation is an over-reaching and imprudent generalization for usage and is beyond the scope of this paper. The null effect of alpha-tocopherol from past studies should encourage researchers to focus more on non alpha-tocopherol vitamins Es; however, not because of the fear of safety issues.

## DELINEATED RESEARCH ON OTHER TOCOPHEROLS, ESPECIALLY DESMETHYL TOCOPHEROLS

Recent findings have called for the reappraisal of the merits of desmethyl tocopherols, especially gamma-tocopherol.[28,29] The following are sample summaries of the research that differentiate C5 desmethyl tocopherols (see hollow arrow in Figure 1) from alpha-tocopherol:

1. Smokers in two groups (Fijians and Cook Islanders) have the same blood levels of alpha-tocopherol, but Fijians with twice the gamma-tocopherol levels (than Cook Islanders) also have 10 times less incidence of lung cancer (than Cook Islanders).[30]
2. Supplementation of diets with alpha-tocopherol reduces serum levels of both gamma- and delta-tocopherols in humans.[26,31]
3. Supplementation of gamma-tocopherol in the diet increased both gamma- and alpha-tocopherol in animals.[32]
4. Epidemiological studies indicated that serum gamma-tocopherol levels correspond to the reduction of prostate cancer [33,34] and coronary heart disease.[35,36,37]
5. In vitro studies support gamma-tocopherol as being more effective than alpha-tocopherol in quenching mutagenic peroxynitrite[38,39] and blocking COX-2 inflammation.[40-42]
6. The uptake of gamma- and delta-tocopherol was much higher than alpha-tocopherol in erythrocytes and macrophages[43,44] which may explain these desmethyl tocopherols' stronger lipid peroxidation protection; and, they may be better tissue markers of oxidative events than alpha-tocopherol.[45,46]
7. In human surgical tissues, there is a higher abundance of gamma-tocopherol to alpha-tocopherol.[44] For example, the gamma/alpha ratios in adipose (31%), vein (33%), muscle (38%), and skin (53%) are much higher compared to those reported in plasma (typically about 10%). Possibly, the bioaccumulation and bioavailability may work through an ATTP-independent pathway.[47]
8. Gamma-tocopherol enters the human brain without discrimination[48] via the blood; however, high alpha-tocopherol in serum tends to suppress gamma-tocopherol in both serum and cerebrospinal fluid.
9. Delta-tocopherol has stronger anti-proliferative effects than alpha- and gamma-tocopherols in rodent mammary neoplasms.[49]

## COMPOSITIONAL VARIATION OF VITAMIN E

Original human studies were performed with tocopherol and tocotrienol mixtures largely derived from palm, the source in which they were first discovered and availed in large abundance.[50,51] Later clinical studies included vita-

min E mixtures from rice and palm (Figure 2). Some clinical reports were equivocal[52,53] because these vitamin E mixtures were high in alpha-tocopherol and alpha-tocotrienol. A recent review suggested that the equivocal anti-lipidemic properties of tocotrienols may be clarified by defined compositions of tocotrienol supplements.[25]

There is a lack of understanding for today's "tocotrienol products." These admixtures are often labeled as tocotrienol-rich fraction (TRF) or full spectrum vitamin E, without regards to the desmethyl tocotrienols (Figure 2). Also, alpha-tocopherol content varies widely. For example, rice and palm "tocotrienols" may contain 25-50% tocopherols, most of which (70 to 90%) is alpha-tocopherol. This is further confounded in that alpha-tocopherol decreases the concentration of alpha-tocotrienol.[54] To summarize, these commercially available TRFs are not uniform, contain little to no delta-tocotrienol, and are high in alpha-tocotrienol and tocopherols, especially alpha-tocopherol. Additionally, because the variations are not properly understood, trade journals often conflate the different vitamin Es when reporting on research results.

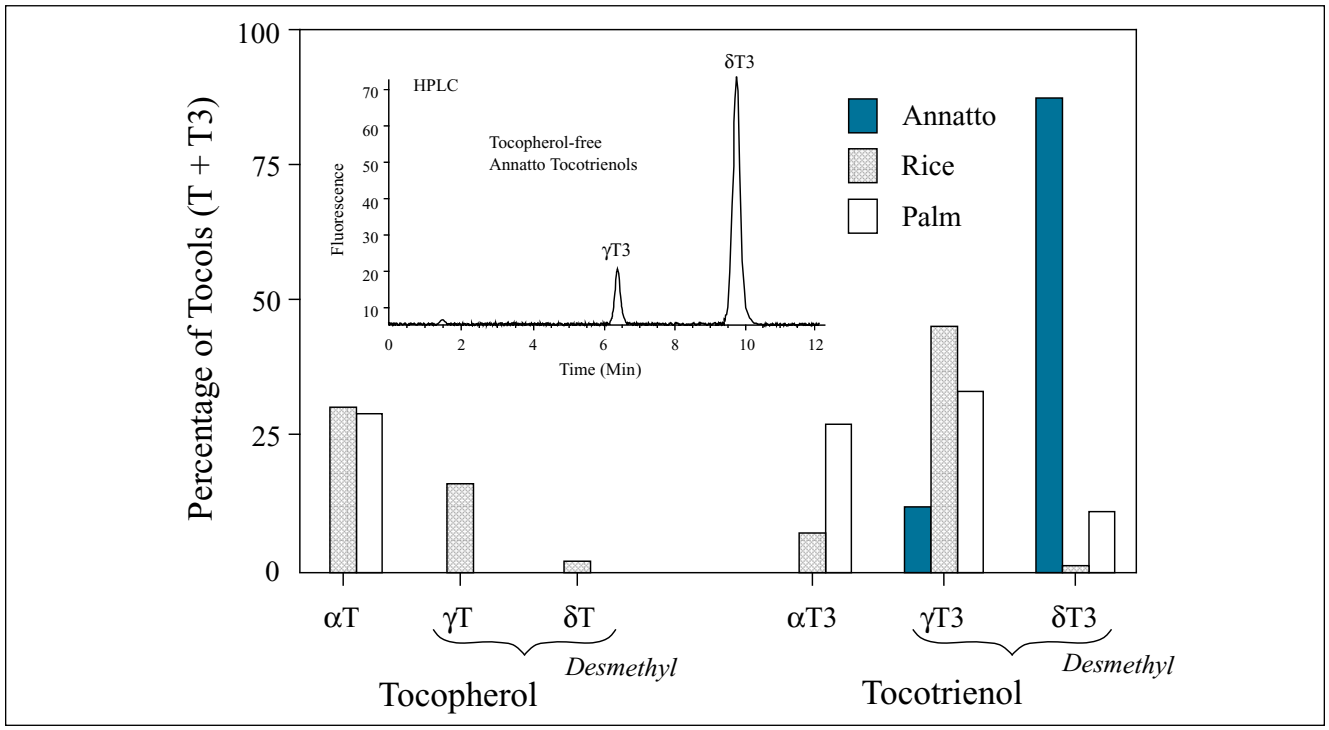
Many "full spectrum" vitamin E products keep the 400 IU (267 mg as RRR- or d-alpha-tocopherol) with about 100 to 200 mg of gamma-tocopherol, and small amounts of tocotrienols (e.g., 5-10 mg). Such "full-spectrum" vitamin formulation is neither consistent with current science nor a reflection of the US diet. In fact, the high alpha-tocopherol is likely to decrease the bioavailability of other vitamin E isomers, including tocotrienols. For instance, one skin formula includes an unspecified 200 to 400 mg tocopherol-tocotrienol blend.[55] Other formulas contain higher tocotrienols (100-200 mg/day; unspecified isomers), mixed tocopherols (about 200 mg/day; unspecified isomers) and alpha-tocopherol (100-250 mg/day) that provide some isomeric balance, but these formulas are still high in non-desmethyl tocopherols.[56,57]

## DELINEATED RESEARCH ON OTHER TOCOTRIENOLS, ESPECIALLY DESMETHYL TOCOTRIENOLS

The following are sample summaries of research that differentiate C5 desmethyl tocotrienols (see hollow arrow in Figure 1) from alpha-tocopherol and alpha-tocotrienol:

1. Delta-tocotrienol and gamma-tocotrienol are potent inhibitors of endogenous cholesterol synthesis.[58,59] Binary mixtures of these desmethyl tocotrienols are synergistic, but combinations with the non-desmethyl alpha-tocotrienol have no additional benefits. Additionally, alpha-tocotrienol is 5-fold less potent than the desmethyl tocotrienols. Tocopherols are inactive in inhibiting cholesterol synthesis.
2. Effective preparations for cholesterol reduction consist of <15% alpha-tocopherol and >60% desmethyl tocotrienols. Less effective (or ineffective) preparations consist of >30% alpha-tocopherol and <45% desmethyl tocotrienols.[60]
3. The cholesterol lowering ability of tocotrienols increases in this order: beta < alpha < gamma < delta < other desmethyls.[61]

**Figure 2:** Composition of plant-derived vitamin Es



4. The cancer lowering ability of tocotrienols increases in this order: alpha<gamma<delta[15,62] where alpha-tocopherol is inactive and delta-tocopherol is weakly active.[49.62]
5. Tocotrienols, especially desmethyl tocotrienols, are more bioavailable than non-desmethyl tocotrienols and tocopherols.[49,64,65]
6. Delta-tocotrienol improves vascular functions, reducing adhesion molecules over that of alpha-tocotrienol.[66]
7. Tocotrienols inhibit angiogenesis (important in tumor growth, diabetic retinopathy, rheumatic arthritis, wet-type macular degeneration) via VEGF inhibition. The ability of tocotrienols increases in this order: alpha < gamma < beta < delta,[67] and tocopherols show very weak inhibition.
8. Delta-tocotrienol and, secondarily, gamma-tocotrienol correct for genetic defects in nerve protein synthesis in children with Familial Dysautonomia, a neurodegenerative genetic disorder.[68] Tocotrienols at concentrations well below their antioxidative properties (4-10x lower) are effective in preventing glutamate-induced neuronal cell deaths.[69,70]
9. Gamma-tocotrienol and alpha-tocotrienol preferentially accumulate in adipose tissue and skin to a similar extent or higher than alpha-tocopherol[54] possibly through an ATTP-independent pathway and bioavailability.[25,47]
10. Other unique effects of tocotrienols, especially the desmethyl isomers, not shared by alpha-tocopherol (e.g., natriuresis, anti-hypertension, anti-inflammation, and anti-osteoporosis) have been reviewed.[1]

## MECHANISMS OF ACTION AND BIOCHEMICAL PATHWAYS

Tocotrienols reduce hepatic cholesterol synthesis by inhibiting the HMG-CoA reductase and by accelerating the degradation of the reductase protein.[59] Tocotrienols also disphosphorylate the farnesyl diphosphate and prevent farnesol reactivation.[72] Two open studies (each with five subjects) were conducted to test the efficacy of C5 desmethyl tocotrienols on lipidemia (satisfying the "requirements" of items 1 to 3 in the section above). Subjects took annatto tocotrienols (75 mg/day), a tocopherol-free, desmethyl tocotrienol-only product, consisting of delta-tocotrienol (typically 90%) and gamma-tocotrienol (typically 10%). On average, the total cholesterol and LDL-cholesterol dropped 13%, triglycerides dropped 23%, and HDL-cholesterol increased modestly by 6% after 2 months of supplementation.[75] These studies are consistent with the mechanism of action and requirement of tocotrienol in order to modulate lipidemia. More controlled clinical studies are being planned to verify these open studies.

Desmethyl tocotrienol applications for cancer chemotherapy and chemoprevention are underpinned by isoprenoid starvation via HMGR inhibition and by cell death via caspase activation.[72,74] Similarly, desmethyl tocopherol (especially gamma tocopherol) applications for cancer chemotherapy and chemoprevention are underpinned with Ras-p21 gene inhibition and PPAR gene activation.[75,76] While there may be additional mechanistic underpinnings that would underscore desmethyl vitamin E in the future, what is clear to date is that alpha-tocopherol does not participate in these mechanisms. Additionally, the evidence is stacked more in favor of desmethyl tocotrienols than desmethyl tocopherols in numerous indications, especially cancer inhibition.

However, it is instructive to know that tocopherols and tocotrienols are themselves undergoing biochemical catabolism in mammals. In the human body, both alpha-tocopherol and gamma-tocopherol undergo enzymatic beta-oxidation for the systematic shortening of the saturated *phytyl* tail to yield the water-soluble urinary carboxyethylhydroxychroman (CEHC) metabolites to the respective alpha-CEHC and gamma-CEHC.[77] Similarly, both alpha-tocotrienol and gamma-tocotrienol also undergo beta-oxidation for the systematic shortening of the unsaturated *farnesyl* tail to yield the same alpha-CEHC and gamma-CEHC, respectively.[78] Recently, delta-tocopherol and delta-tocotrienol were also added to the list that yields the analogous delta-CEHC.[71,79] Therefore, catabolically the respective tocopherols and tocotrienols convert to their CEHCs. However, only the desmethyl CEHCs have benefits of sodium excretion, reduction of body fluid retention and hypertension,[80,81] inhibition of prostate cancer,[82] and suppression of TNF-alpha-induced activation of microglial cells.[83]

## MINING VITAMIN E FOR HUMAN HEALTH

North Americans consume some 70% of vitamin E as gamma-tocopherol because of the abundance of soy and corn derived products in the diet.[84] Vitamin E intake can be obtained from USDA databases.[85] US daily dietary consumption of vitamin Es may be broken down to 18 mg gamma-tocopherol,[86] 10-12 mg alpha-tocopherol, and 7-10 mg of all other tocols (approximately equally 2-4 mg each of tocotrienols combined, delta-tocopherol, and beta-tocopherol), providing a possible total of 35-40 mg per day of all E vitamins as daily consumption (DC). All 4 tocotrienol isomers (Figure 1) were identified in US foods.[2,85] Therefore, a vitamin E supplement that reflects the diet should predominate in gamma-tocopherol (where the gamma : alpha ratio is 3:1 to 4:1), and contain about 8% as tocotrienols. A proper description for this dietary vitamin E supplement should be "appropriate spectrum E" instead of the familiar "full spectrum E". "Appropriate spectrum E" will more closely reflect the composition of our diet, and is therefore well suited for *maintenance* (perhaps 35-40 mg/day = 1X DC) or *prevention* (perhaps 10X DC).

Appropriate spectrum E could take a form that is suit-

able for therapy where tocotrienol concentration is significantly higher than tocopherol. The study summaries above argue for a vitamin E that contains desmethyl tocotrienols. Exemplary formulations for therapy should contain some 80% desmethyl vitamin Es and the tocopherol-to-tocotrienol ratio should not exceed 1:4 to maintain the effectiveness of tocotrienols.[87] Such appropriate spectrum E for *treatment* (perhaps 50-200 mg/day as desmethyl tocotrienols) may be supplemented with other non vitamin E nutrients, especially for cardiovascular, diabetes, and cancer applications.

## CONCLUSIONS

Past publications of composition data of whole and process foods typically include alpha-tocopherol and occasionally desmethyl tocopherols. There is a prevailing bias towards alpha-tocopherol as vitamin E given the absence of tocotrienols in published composition data. Since each of the four commonly occurring isomers of tocotrienols and of tocopherols exhibits varying degrees of activity and efficacy, the product standardization is critical when formulating an optimal vitamin E admixture. The generalized order of potency for tocotrienol is: delta-tocotrienol ≥ gamma-tocotrienol > alpha-tocotrienol, and that of the tocopherol series is desmethyl tocopherols > alpha-tocopherol. This potency ranking holds true for indications including cancer inhibition, endothelial functions (i.e., inhibition of platelet aggregation and adhesion molecules), nerve function correction/protection, anti-angiogenesis, sodium excretion, and anti-hypertension. In addition to potency, an "appropriate spectrum" vitamin E must also consider application specific synergism and interference existing among the various vitamin E isomers.

Over-emphasis of alpha-tocopherol is a result of several factors including: a) its illustrious introduction to the masses that littered the popular and scientific literature, b) inordinate IU claims motivated by market-driven sales, and c) the disconnect in the definition of RDA (based on hemolysis prevention and/or rat fetal resorption assay), leading to confusion in the perceived antioxidant value. This paper concludes that alpha-tocopherol should be de-emphasized and a higher priority be given to desmethyl vitamin Es, especially desmethyl tocotrienols, whose therapeutic functions are unshared by or superior to alpha-tocopherol.

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# C-Reactive Protein (CRP) – A New Blood Marker for Coronary Vascular Disease

## Should you be testing the CRP levels of patients who are at risk for CVD?

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As inflammation has come to be recognized as a key factor in the atherothrombotic process,[1,2] interest in methods of measuring that inflammation and using the results to predict the risk of cardiovascular disease (CVD) has grown. Markers of inflammation such as fibrinogen and the white blood cell count have been linked to CVD,[3] but none had proven to be reliable and affordable predictors of cardiovascular risk.

In recent years, studies have demonstrated that C-reactive protein (CRP), "a pentameric protein associated with inflammation"[4] that has been used to monitor rheumatologic conditions, is an effective indicator of both inflammation in the body and the risk of CVD and associated ailments. A 2001 editorial titled "The renaissance of C reactive protein," published in the *British Medical Journal*, cited studies showing that "Increased C reactive protein values significantly predict coronary events in outpatients with stable or unstable angina and in hospital patients with severe unstable angina, and predict outcome after coronary angioplasty."[5] The authors of this article noted that the levels of CRP in healthy people could help predict the risk of future coronary events.

Since then, additional studies have strengthened the link between CRP levels and CVD and associated ailments. Here, for example, are summaries of some more recent CRP research, taken from the American Heart Association's web site:

- "In a group of more than 27,000 women participating in the ongoing Women's Health Study, elevated levels of C-reactive protein (CRP), a blood-marker for inflammation, was a better predictor of risk for future cardiovascular (CVD) events than elevated low-density lipoprotein (LDL) cholesterol levels."[6]
- "In women with metabolic syndrome, blood levels of C-reactive protein (CRP) can help predict cardiovascular risk, researchers reported today in *Circulation*…"[7]
- "High levels of the inflammation marker C-reactive protein (CRP) in healthy, middle-aged men signals an increased risk of ischemic stroke in later life, according to a 20-year follow up study reported in today's rapid access issue of *Circulation*…"[8]
- "Elevated levels of a blood-marker for inflammation [CRP] are associated with a significantly increased risk of stroke among the elderly regardless of the amount of plaque in the arteries leading to the brain, scientists report in today's rapid access issue of *Circulation*…"[9]
- "People with a high level of C-reactive protein (CRP), a marker of inflammation in the body, don't receive the same beneficial reductions in cholesterol while on a low-fat, low-cholesterol diet as those with lower levels of CRP, according to a study in today's rapid access issue of *Circulation*…"[10]

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• "High blood pressure and elevated levels of the inflammation marker C-reactive protein (CRP) may work together to increase cardiovascular risk in women, according to a study in today's rapid access issue of *Circulation*…"[11]

Writing in *Current Problems in Cardiology* in 2004, Paul M. Ridker, MD, MPH, and his collaborators from Brigham and Women's Hospital in Boston argue that high-sensitivity CRP "predicts incident myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death among healthy individuals with no history of cardiovascular disease, and recurrent events and death in patients with acute or stable coronary syndromes."[12]

Two brand new studies strengthen the link between CRP and CVD. The first, conducted by Paul Ridder, MD, and his colleagues from Brigham and Women's Hospital and Harvard Medical School, looked at the link between CRP and LDL levels after treatment with statin drugs, and the risk of recurrent heart attack or death from coronary disease in 3,700+ people with acute coronary syndromes.[13] The researchers concluded that "Patients who have low CRP levels after statin therapy have better clinical outcomes than those with higher CRP levels, regardless of the resultant level of LDL cholesterol."

In the second study, researchers from the Cleveland Clinic used intravascular ultrasonography to track the development of coronary disease in 502 patients receiving statin therapy.[14] They noted that "Patients with reductions in both LDL cholesterol and CRP that were greater than the median had significantly slower rates of [atherosclerosis] progression than patients with reductions in both biomarkers that were less than the median."

**Using hsCRP**

The CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease met in March, 2002, to study the types and uses of inflammatory markers as predictors of CVD risk. The Clinical Practice Discussion Group offered these, among other, recommendations for clinical practice:[15]

• "High-sensitivity C-reactive protein (hsCRP) is an independent marker of risk" for CVD. At their discretion, physicians may use hsCRP in patients felt to be at "intermediate risk (10% to 20% risk of coronary heart disease per 10 years) for cardiovascular disease…"

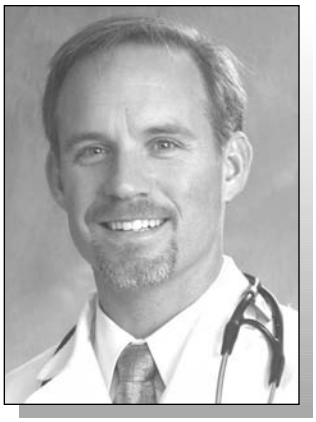
• "hsCRP is an independent marker of risk" even in apparently healthy adults. It may be "used at the discretion of the physician as part of a global coronary risk assessment in adults without known CVD."

• "hsCRP measurement in patients with stable coronary disease or acute coronary syndromes (ACS) may be useful as an independent marker of prognosis for recurrent events, including death, myocardial infarction, and restenosis after percutaneous coronary intervention (PCI)."

The members of the Clinical Practice Discussion Group note that the benefits of treatments based on hsCRP measurements are still "uncertain," and recommend research to fill in the blanks in our knowledge.

As for interpreting the results of hsCRP tests, levels less than 1 are associated with lower cardiovascular risk; between 1 and 3 with moderate risk; and above 3 with higher risk.[16] Repeated readings of 10 or more should be "evaluated for noncardiovascular causes."[17]

**Lowering CRP**



**Tim Church, MD**

According to Tim Church, MD, Medical Director of the Cooper Institute in Dallas, Texas, there are only a few recognized methods of lowering CRP levels. Statin drugs work for some people. Taking aspirin, exercising, going on the Mediterranean Diet, and weight loss for the overweight can also lower levels of this inflammation marker. As the results of Dr. Church's study published in 2003 in the *American Journal of Medicine* indicate, vitamins can also be helpful.[18] Dr. Church and his collaborators had already conducted a study showing that "a multivitamin with antioxidant properties could reduce the oxidation of LDL cholesterol, and could also lower homocysteine. We also knew, from the work of other researchers, that vitamin E could lower the C-reactive protein levels in people with diabetes. So we decided to see whether multivitamins could lower C-reactive protein. We went back to the frozen blood samples from our original study and found that taking multivitamins, specifically $B_6$ and C, was associated with a 14% drop in CRP."

**A Caution on Measuring CRP**

Dr. Church cautions that testing CRP levels can be "tricky," for levels can move up and down in response to levels of inflammation unconnected to CVD. For example, "you may see acute spikes with a sinus infection, or an arthritic flare up. CRP should be treated like blood pressure readings; you need multiple measures to determine a person's baseline."

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ORIGINAL RESEARCH

# Enhancement of Cardio-Protective Effects and Attenuation of Adverse Effects of Female Sex Hormones on Cultured Human Vascular Smooth Muscle Cells by a Combination of Ascorbic Acid, Lysine, Proline, Arginine, Cysteine, and Epigallocatechin Gallate

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**ABSTRACT**

In this *in vitro* study, the effects of adjunctive use of a formulation containing ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate (NS) with female sex hormones were tested on human aortic smooth muscle cells (SMC). Estradiol and progesterone stimulated DNA synthesis in SMC 30% and 24% respectively at 25–150 nmol/L concentrations. NS (20 µg/ml) inhibited SMC growth by 30% over the control, and reversed the stimulatory effect of the sex hormones to a maximum of 25% inhibition. Dehydroepiandrosterone sulfate (DHEAS) inhibited SMC growth by 50% at 0.1 mmol/L. Addition of NS enhanced the DHEAS inhibitory effect to 70% as compared to the control. DHEAS and progesterone significantly increased SMC capacity to invade Matrigel by 20% and 60%, respectively. Addition of NS reversed the stimulatory effects, producing up to 60% inhibition of SMC invasion. Addition of NS reversed the effects of DHEAS on total collagen synthesis in SMC from 28% stimulation to 56% inhibition. Estradiol, progesterone, and DHEAS demonstrated some inhibition of tumor-necrosis-factor-alpha-stimulated SMC secretion of interleukin (IL) 1-beta, IL-6, and monocyte chemo attractant protein 1 in cultured media; NS enhanced inhibition of these cytokines under most conditions. The results of this study imply that the specific formula of nutrients tested enhances the cardio-protective effects of female sex hormones and counteracts their adverse effects on atherogenic properties.

**KEYWORDS**: cardiovascular, estradiol, progesterone, DHEAS

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## INTRODUCTION

Large numbers of menopausal women are using hormone replacement therapy (HRT) with different forms of estradiol either taken alone or supplemented with progesterone for counteracting such adverse effects as hot flashes, sudden mood changes, loss of bone mass, and others. While estradiol has been shown to exert some protective effects on the cardiovascular system, such as suppression of vascular monocyte chemotactic protein-1 expression during early atherogenesis,[1] hormone replacement therapy has produced major adverse effects that far outweigh these limited benefits. After a mean of 5.2 years of follow-up, the estrogen and progesterone versus placebo arm of the Women's Health Initiative study of the risk of cardiovascular disease was stopped because of adverse cardiovascular effects—higher rates of coronary heart disease (CHD), stroke, and venous

thrombosis among women taking estrogen and progesterone compared with placebo.[2] In addition, recent studies evaluating the effect of estrogen alone and with progesterone on both coronary artery stenosis and carotid intima medial wall thickness have also shown little benefit from hormone therapy.[3] Due to these and additional adverse effects, such as increased potential of developing breast cancer, there is an urgent need for effective alternative therapy.[4]

The formation of an atherosclerotic lesion is associated with drastic behavioral modifications by arterial wall smooth muscle cells (SMC), including: massive migration of SMC from the vascular medial to the intimal layer, dedifferentiation of SMC to proliferating phenotype, and increased secretion of inflammatory mediating cytokines, as an autocrine response to inflammatory stimuli. These events trigger vessel wall thickening and monocyte recruitment from blood, and lead to progression of the atherogenic cascade. Pathogenic changes of the blood vessel wall in atherosclerosis are accompanied by neointimal thickening resulting from the increased deposition of extracellular matrix proteins by smooth muscle cells that migrate and proliferate in the affected blood vessel areas.[5] Various patho-physiologic events can promote this process, such as inflammation involving local secretion of inflammatory mediators (cytokines), oxidative processes accompanying low-density lipoprotein and lipoprotein(a) deposition, and intracellular-membrane-mediated events, such as changes in protein kinase C activity.[6-7] Various matrix components also affect cellular proliferation, differentiation, and expression of specific genes.[8]

The extracellular matrix (ECM) serves as a reservoir to which various growth factors may be bound, affecting vascular cell growth, differentiation status, and ECM production. Thus, factors affecting matrix components may influence various metabolic processes that accompany initiation and progression of atherosclerosis.

Rath et al proposed that chronic sub-clinical vitamin C deficiency is a primary cause for atherosclerotic plaque formation, as it leads to deposition of lipoprotein(a) and fibrinogen/fibrin in the vascular wall.[9] Ascorbic acid is essential to the synthesis and maintenance of collagen, which maintains blood vessel wall stability. Prolonged deficiency of ascorbic acid, a nutrient not produced in the body, hinders the enzymatic hydroxylation of proline and lysine residues in collagen molecules, thereby weakening the stability of the vascular wall. Thus, combined supplementation with ascorbic acid and lysine has been proposed as a preventive measure to atherosclerosis development.[9-10] A previous study of the direct and matrix-mediated effects of ascorbate on the proliferation rate of vascular smooth muscle cells (VSMC) isolated from guinea-pig aorta, revealed diminished cell proliferation in the presence of 0.5 – 2 mM ascorbate, in a dose-dependent manner without cytotoxic effect.[11] Additionally, a number of studies show cardio-protective effects, through potent antioxidant activity, from chronic tea consumption.[12]

Naturally occurring compounds demonstrate wider spectra of biological activity and fewer side effects than synthetic drugs. It is known that a mixture of natural compounds often produces a synergistically-enhanced therapeutic effect. The objective of this study was to investigate whether a specific formulation of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate (from green tea extract) would modulate the cardiovascular effects of 17-b estradiol and progesterone, using the cultured vascular smooth muscle cell model.

## METHODS AND MATERIALS

**Materials:** Tissue culture plastics were obtained from Becton Dickinson, (San Jose, CA ). Tissue culture supplies (growth media, antibiotics, and trypsin-EDTA) were obtained from Life Technologies, (Grand Island, NY). Fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD). Scintillation fluid BetaBlend and (methyl-3H) Thymidine (25 Ci/mole) were from ICN Biomedicals (Costa Mesa, CA, USA). L-ascorbic acid, bovine serum albumin (fraction V) (BSA), 17-beta estradiol, progesterone, and other chemicals were from Sigma-Aldrich, (St. Louis, MO).

**Cell Culture:** Cell cultures of human aortic smooth muscle cells (SMC) were obtained from BioWhittaker. SMC were cultured in Dulbecco's modified Eagle medium (hereafter DMEM), supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS (v/v) at 37°C in a humidified atmosphere containing 5% $CO_2$, and were split 1:3 to 1:5 upon reaching confluence. SMC at passages 5–8 were used in experiments.

**Cell proliferation assay:** SMC proliferation was assayed by (3H)-thymidine incorporation into cellular genetic material. Cells were plated in 24-well plates at a density of 10,000 cells per $cm^2$ in 0.5 ml of DMEM supplemented with 2% FBS. The attached cells were supplied every 24 hours with fresh growth medium plus additions, as specified in the protocols. A stock solution of NS (composed of vitamin C 700 mg, L-lysine 1000 mg, L-proline 750 mg, L-arginine 500 mg, N-acetyl-cysteine 200 mg, and standardized green tea extract (80% polyphenol, 20% EGCG) 1000 mg) was prepared daily immediately before addition to cell cultures by dissolving in DMEM to a concentration of 10 mg/ml, vigorously vortexed for 1 minute, and filtered through a 0.2 ?m sterile filter. Cell proliferation was measured 3 days later by the addition of 1 µCi/ml (3H)-thymidine to the cell culture for the last 24 hours of the experiment. Cells were washed three times with cold phosphate-buffered saline (PBS), pH 7.2, incubated with 10% trichloracetic acid for 15 minutes at 4°C, washed with cold ethanol, air-dried, solubilized in 0.5 N sodium hydroxide, and then neutralized with hydrochloric acid. Samples were mixed with scintillation fluid and counted using a liquid scin-

tillation counter (model 6500 LS, Beckman Instruments, (Porterville, CA). Cellular DNA-incorporated radioactivity was expressed as d/min per well.

**Cell invasion assay:** SMC cultures grown in 75 $cm^2$ flasks were metabolically labeled by incubating in growth medium containing 1 µCi/ml (3H) thymidine for 24 hours. Cells were washed with PBS, suspended by trypsinization in DMEM supplemented with 10% FBS, and placed on upper surface of porous membrane (3 µm pores) covered with Matrigel (Becton Dickinson). After cell attachment (1.5-2 hours) the cell culture medium was replaced by serum-free DMEM supplemented with tested compounds, as indicated. 10 ng/ml basic fibroblast growth factor (Clonetics Walkersville, MD) was added to the lower chamber. After 24 hours, incubation inserts were removed from the plate and extensively washed with PBS. SMC invasion was estimated by counting radioactivity present on the lower surface of the porous membrane after removal of cells remaining on the upper surface of the insert with cotton tissue.

**Collagen synthesis**: SMC were placed on 24-well plates and grown as a monolayer to confluence for 5–7 days, at which point growth media were replaced with DMEM containing 2% FBS and indicated compounds. Cells were incubated for 3 days with fresh media added daily. For the last 24 hours of incubation, 1 µCi/ml (3H) proline (Sigma-Aldrich) was added to the media. Cell layers were washed three times with PBS and solubilized with 0.5N sodium hydroxide for 18 hours at 60°C. Collagen synthesis was estimated as radioactivity retained within the cell/extracellular matrix layer.

**Cytokine secretion:** SMC were placed on 24-well plates and grown to a confluent monolayer. 5–7 days later, the growth media was replaced with serum-free DMEM and treated with NS. After 24 hours, the media were replaced with fresh media containing the same compounds and tumor necrosis factor alpha (TNFα, Sigma-Aldrich). After 24 hours incubation, the conditioned media were harvested for analysis and assayed for indicated cytokine presence using Quantikine ELISA kits (R&D Systems (Minneapolis, MN ) according to manufacturer's protocols.

**Test Reagents:** In the design of the experiment, a stock solution of a nutrient mixture (NS), weighing 4.4 gm, was prepared daily to treat the cells, composed of the following nutrients: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1 mg.

**Statistical Analysis:** All experiments were performed at least twice with cells from different passages and originating from different donors. The data represent the average (± S.D.) from representative experiments performed in three or more replications. Student's t-test was used for data comparison.

## RESULTS

### Effect of female sex hormones and NS on SMC growth

Estradiol and progesterone-stimulated DNA synthesis was increased by 24% and 30% respectively at progesterone and estradiol concentrations of 150 nmol/L (Figures 1A-B). Cell growth stimulatory effects were attenuated with hormone concentrations increased to 450 nmol/L. NS inhibited SMC growth by 30% at 20 µg/ml (corresponding ascorbic acid content was 20 mmol/L) when used individually, and reversed the hormone–associated stimulatory effect to inhibitory (25% maximum inhibition) when used with estradiol or progesterone. Dehydroepiandrosterone sulfate (DHEAS), a potential metabolic precursor of estrogen, inhibited SMC growth by 48% at 0.1 mmol (Figure 1C). Addition of NS further enhanced the DHEAS inhibitory effect to 68% inhibition as compared to the control.

### Effects of female sex hormones and NS on SMC Matrigel invasion

DHEAS and progesterone significantly increased SMC Matrigel invasion by 37% and 54% respectively (Figure 2). SMC Matrigel invasion was significantly inhibited in the presence of NS when tested with all three of these female sex hormones: estradiol by 67% ($p<0.01$), progesterone by 77% ($p<0.01$), and DHEAS by 78% ($p=0.03$).

### Effects of female sex hormones and NS on total collagen synthesis by SMC

DHEAS increased SMC collagen synthesis by 28%. Addition of NS reduced collagen synthesis in the presence of all sex hormones studied, reversing the effects of DHEAS on SMC total collagen synthesis from 28% stimulation to 56% inhibition as compared to the control (Figure 3). FSH, LH, estradiol, and progesterone did not affect collagen synthesis when used individually.

### Effects of female sex hormones and NS on the secretion of inflammatory mediators by SMC

Estradiol, progesterone, and DHEAS slightly inhibited (up to 20%) tumor-necrosis-factor-alpha stimulated SMC secretion of the inflammation mediators: interleukin 1-beta and interleukin-6 in culture media. The inhibitory effect was further enhanced by addition of NS. DHEAS inhibited TNFa-stimulated secretion of monocyte chemoattractant protein; however, NS did not enhance this effect.

## DISCUSSION

The results from this study demonstrated that NS attenuated the pro-atherogenic modification of SMC physiological properties, such as increased growth rate and invasiveness, excessive production of extracellular matrix components, and autocrine production of inflammatory cytokines, stimulated by female sex hormones (estradiol, progesterone, and dehydorepiandrosterone sulfate). Estradiol and progesterone were found to stimulate SMC growth at 50

**Figure 1A:** Effect of estradiol independently and in the presence of the nutrient mixture (NS 20mcg/ml) on aortic SMC DNA synthesis (as % of control) tested at 0, 50, 150, and 450 nM concentrations of estradiol.

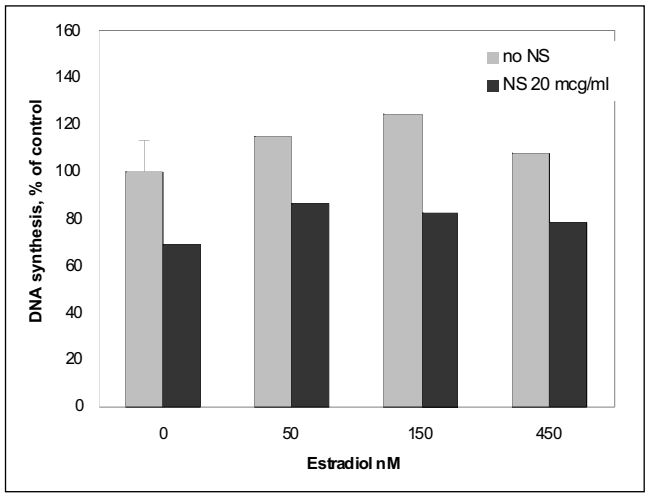



**Figure 1B:** Effect of progesterone independently and in the presence of the nutrient mixture (NS 20 mcg/ml) on aortic SMC DNA synthesis (as % of control) tested at 0, 50, 150, and 450 nM concentrations of progesterone.

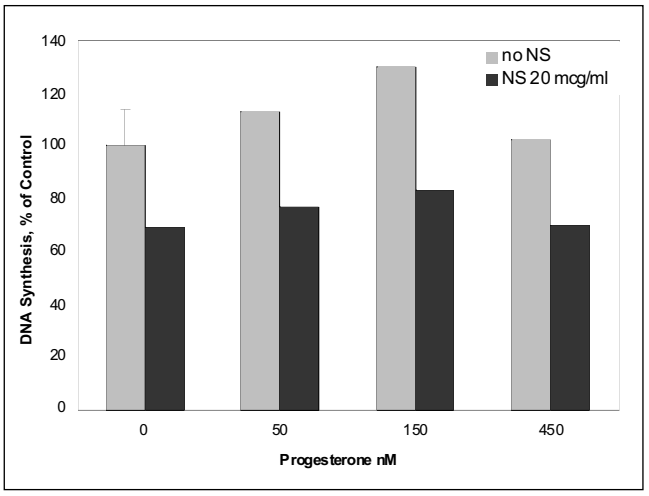



**Figure 1C:** Effect of NS (mcg/ml) and DHEAS on aortic SMC DNA synthesis (as % of control) tested at 0, 25, 50, and 100 mcM concentrations of DHEAS.

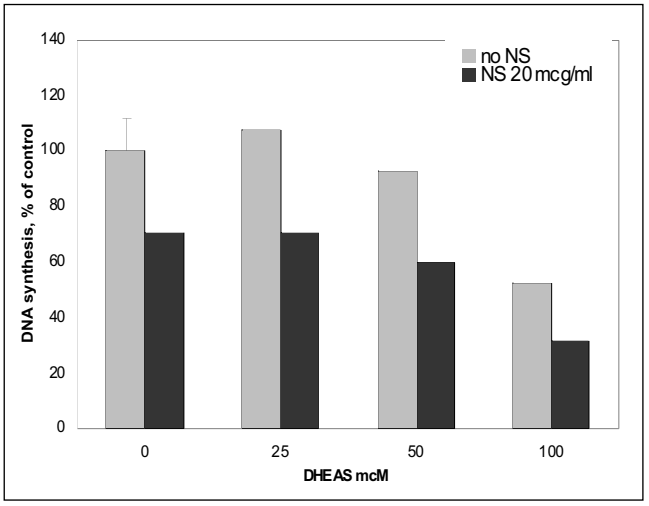
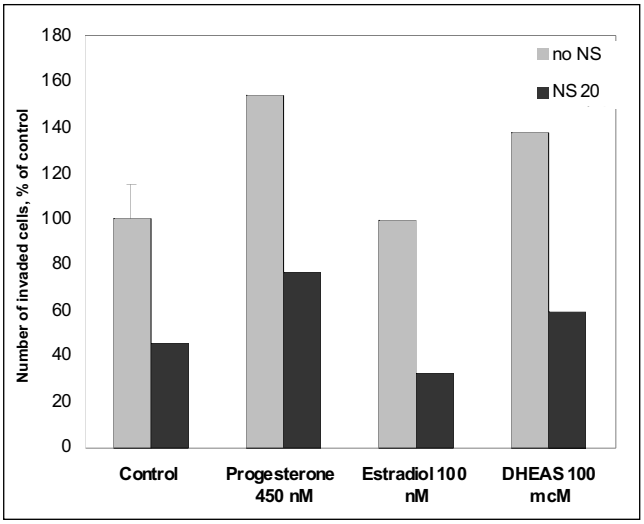



**Figure 2:** Effect of estradiol, progesterone, and DHEAS on SMC invasion through extracellular matrix (as % of control) tested independently and in the presence of NS 20 mcg/ml.




and 150 nm, which was attenuated at all concentrations in the presence of NS 20 µg/ml. These results contradict previously reported anti-mitotic and anti-migrating effects of estradiol on cultured porcine SMC.[13] Though DHEAS did not stimulate SMC proliferation, NS showed inhibition of SMC proliferation at all concentrations of DHEAS. Both progesterone and DHEAS significantly increased SMC invasiveness. Though estradiol did not stimulate SMC invasion at 100 nM, SMC Matrigel invasion was significantly inhibited in the presence of NS when tested with all three of these female sex hormones:estradiol by 67% ($p<0.01$), progesterone by 77% ($p<0.01$), and DHEAS by 78% ($p=0.03$).

Furthermore, NS inhibited the stimulatory effect of DHEAS on collagen synthesis. Thus, the synergistic activity of the specific nutrient mixture significantly enhanced vascular wall stability as evidenced by the significantly decreased SMC Matrigel invasion. Stability of the vascular wall is dependent upon presence of sufficient quantities of ascorbic acid, lysine, and proline for synthesis of optimal collagen structure, as discussed previously. Various studies have shown that restructuring of the vascular matrix is facilitated by ascorbate, pyridoxine, L-lysine, and L-proline.[14-15]

The inhibitory effects of other individual nutrients composing NS have been reported in both clinical and experimental studies. Anti-atherogenic effects of green tea extract have been demonstrated in animal and *in vitro* studies. For example, apoprotein E-deficient mice fed green tea for 14 weeks had attenuation of aortic atheromatous areas by 23%

**Figure 3:** Effect of various female sex hormones independently and in the presence of 100 mcg/ml of the nutrient mixture (NS) on aortic SMC collagen synthesis (as % of control).

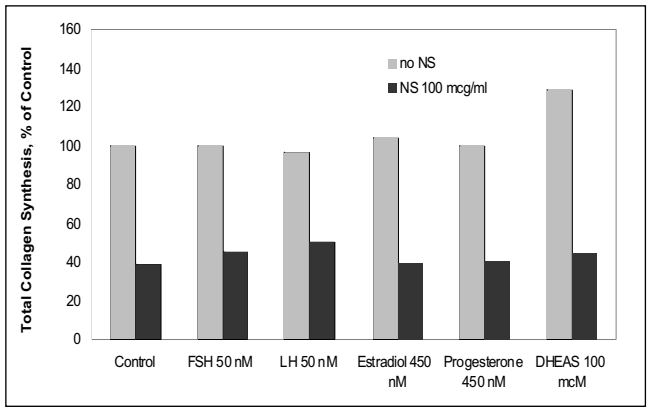



**Figure 4A:** Effect of TNFa and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of interleukin-1beta.

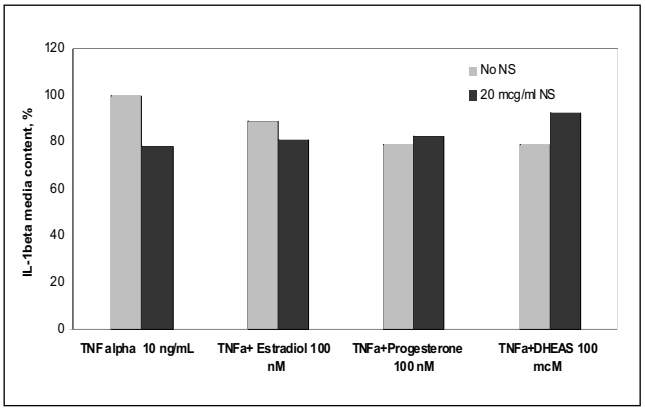



**Figure 4B:** Effect of TNF-alpha and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of interleukin-6.

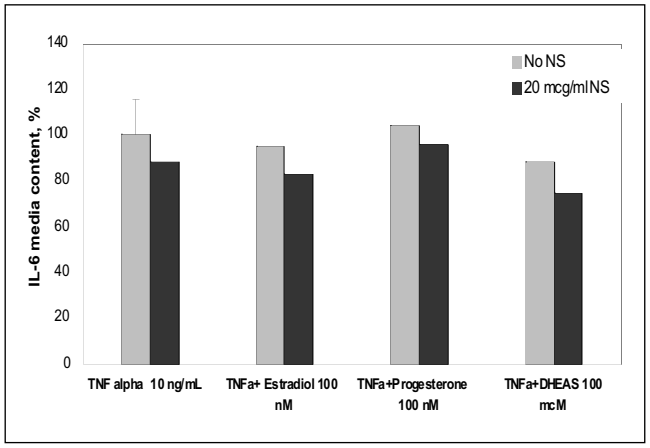
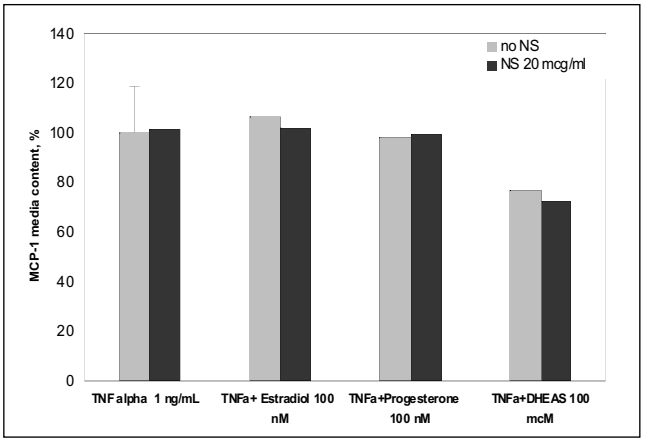



**Figure 4C:** Effect of TNF-alpha and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of monocyte chemoattractant protein-1(MCP-1).




and decreased aortic cholesterol and triglyceride levels over the control group.[12] Furthermore, epidemiological and clinical studies have documented the benefits of individual nutrients in prevention of cardiovascular disease.[16-17]

However, individual nutrient effects have been shown to be enhanced when acting in synergy. Our previous studies demonstrated that the anticancer effect of ascorbic acid, proline, lysine, and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients.[18] Furthermore, cardio-protective effects of NS were confirmed in our study of the effect of nutrient supplementation in progression of early coronary atherosclerosis. In this pilot study, the extent of coronary calcification in 55 patients diagnosed with early coronary atherosclerosis was measured prior to nutrient supplementation and after one year of intervention, using an Imatron C-100 Ultrafast CT scanner.[19] Progression of coronary calcification, as determined by the CAS score, decreased significantly (from 0.49 $mm^2$ to 0.28 $mm^2$ monthly growth) after one year of nutritional intervention.

## CONCLUSION

While clinical trials are necessary to examine the full cardio-protective benefits of the combination of nutrients tested, the results of this study imply that the specific combination of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate tested enhances the protective effects of estradiol, progesterone, and DHEAS on the cardiovascular system and inhibits the adverse effects these hormones have on atherogenic properties. These results, coupled with prior research studies, demonstrating the cardio-protective properties of the synergistic nutrient combination of ascorbic acid, lysine, proline, arginine, and EGCG, support its potential as a strong candidate in the prevention of cardiovascular disease.

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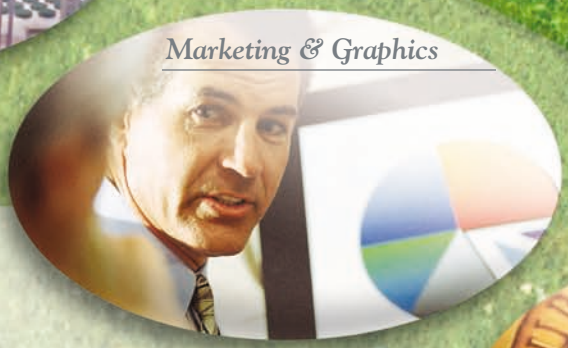

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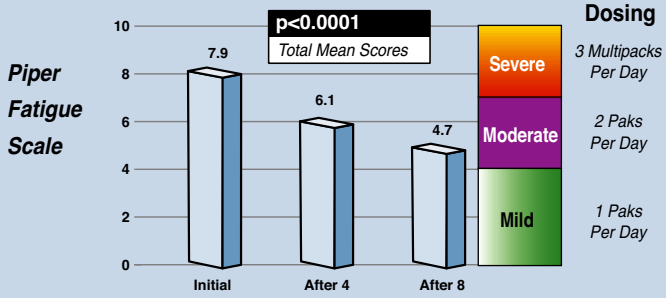


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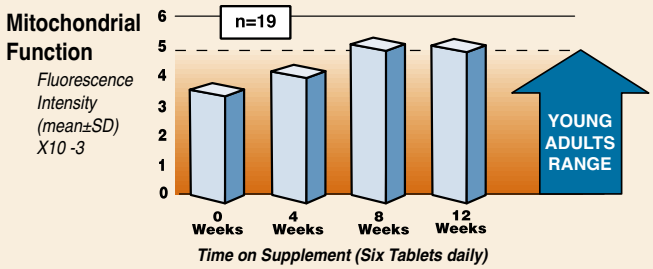


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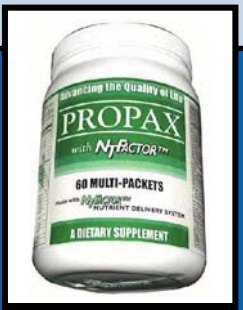


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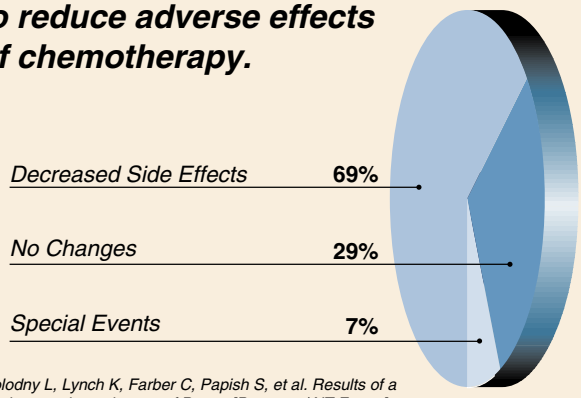
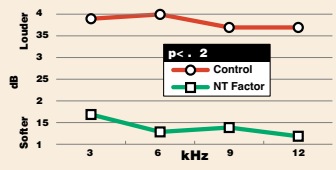
## Results of a Double Blinded Randomized Crossover Placebo Controlled study to evaluate the use of Propax w/ NT Factor to reduce adverse effects of chemotherapy.




Colodny L, Lynch K, Farber C, Papish S, et al. Results of a study to evaluate the use of Propax[Propax w/ NT Factor] to reduce adverse effects of chemotherapy. JANA 2000; 2: 17-25. Results of a Double Blinded Randomized Crossover Placebo Controlled study to evaluate the use of Propax w/ NT Factor to reduce adverse effects of chemotherapy

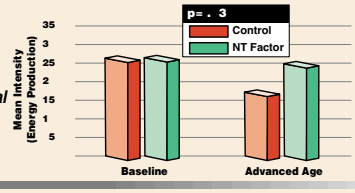
## Pre-Clinical Publications

**Better Able to Hear Sounds; Results of Auditory Threshold Shifts**



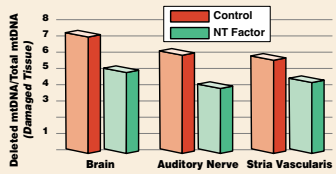

Hearing threshold shifts at six months from baseline values. Statistically significant preservation of thresholds is noted with NT Factor™. Animals on NT Factor™ Diet continue to hear sounds at a lower volume.

**Less Age-Related Loss of Mitochondrial Function (Energy)**




Mitochondrial membrane function/potential was measured by intensity of uptake by Rhodamine 123.

**Lower Ratio of Oxidative Damage mtDNA Quantification (Free Radical Damage)**




Mitochondrial DNA deletion quantification demonstrates a lower ratio of common aging deletion (mtDNA4834) in the NT Factor™ group as opposed to the control group.

Seidman M, Khan MU, Tang W, Quirk WS, Otolaryncology Head & Neck Surgery; Sept. 2002;pg 138-44
Seidman M, Anti-Aging Medical News, Winter 2000-01; pg 5, 16, 32, 44