Use of glucosamine, chondroitin, and omega-3 fatty acid supplements in relation to inflammation and risk of colorectal cancer

Elizabeth D. Kantor

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Reading Committee:

J Emily White, Chair

Johanna Lampe

Ulrike Peters

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Abstract

Use of glucosamine, chondroitin, and omega-3 fatty acid supplements in relation to inflammation and risk of colorectal cancer

Elizabeth D. Kantor

Chair of the Supervisory Committee:

Professor J Emily White

Department of Epidemiology

In a prior exploratory analysis conducted within the VITamins and Lifestyle (VITAL) cohort study, we observed that use of glucosamine, chondroitin, and omega-3 polyunsaturated fatty acid (omega-3 PUFA)-containing fish oil supplements was associated with decreased risk of colorectal cancer (CRC) after 5 years of follow-up. With an additional 2 years of follow-up in the VITAL cohort, we have examined these associations in more detail among 77,719 adults aged 50-76, and have explored the biologic mechanisms by which these supplements may reduce CRC risk. Data on 220 VITAL biomarker study participants was used to test whether use of glucosamine, chondroitin, and fish oil supplements is associated with oxidative stress, DNA damage, and DNA repair capacity. Additionally, we have used data from the National Health And Nutrition Examination Survey (NHANES) to evaluate the association between use of these supplements and inflammation among 9,947 adults aged 25 and older.

Persons reporting use of glucosamine+chondroitin on 4+ days/week for 3+ years had 45% lower risk of CRC than non-users [Hazard Ratio (HR): 0.55; 95% CI: 0.30-1.01; p-trend: 0.16]. This association varied by

body mass index (p-interaction: 0.006), with a significant inverse association observed among the overweight/obese only. As compared to non-use, high use of fish oil supplements (4+ days/week for 3+ years) was associated 49% reduced of CRC (HR: 0.51; 95% CI: 0.26-1.00; p-trend: 0.06). The association between fish oil use and decreased risk of CRC was primarily observed among men (p-interaction: 0.02), and for cancers of the colon rather than cancers of the rectum (p-difference: 0.05). We also examined the associations between omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and CRC, as well as between dark fish consumption and CRC. While total (diet+supplementary) EPA+DHA intake and dark fish consumption were not associated with CRC overall, these associations were modified by underlying genetic risk (p-interaction: 0.009 and 0.02, respectively): significant inverse associations were observed among persons of low and moderate genetic risk, while positive associations were observed among persons of high genetic risk.

Glucosamine and chondroitin supplements were also observed to be associated with reduced oxidative stress, as measured by prostaglandin 2 alpha (PGF2α; p-trend: 0.01 and p-trend: 0.003, respectively) and reduced inflammation, as measured by high-sensitivity C-reactive protein (hsCRP). Persons reporting use of glucosamine supplements experienced 17% lower hsCRP than non-users (0.83; 95% CI: 0.74-0.93), while chondroitin users had 22% lower hsCRP than non-users (0.78; 95% CI: 0.67-0.92). However, use of glucosamine and chondroitin supplements was not associated with DNA damage, DNA repair, or 8-isoprostane, another measure of oxidative stress. Fish oil supplement use was associated with reduced hsCRP (ratio: 0.84; 95% CI: 0.71-1.00), but not with measures of oxidative stress/DNA damage.

Our findings suggest that use of glucosamine, chondroitin, and fish oil supplements may be associated with a reduced risk of CRC and offer plausible biologic mechanisms to support these observed associations. CRC poses a substantial health burden in the United States, and there is great need to

identify safe and effective preventives. Further research will be needed to better understand the chemopreventive potential of these supplements.

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INTRODUCTION

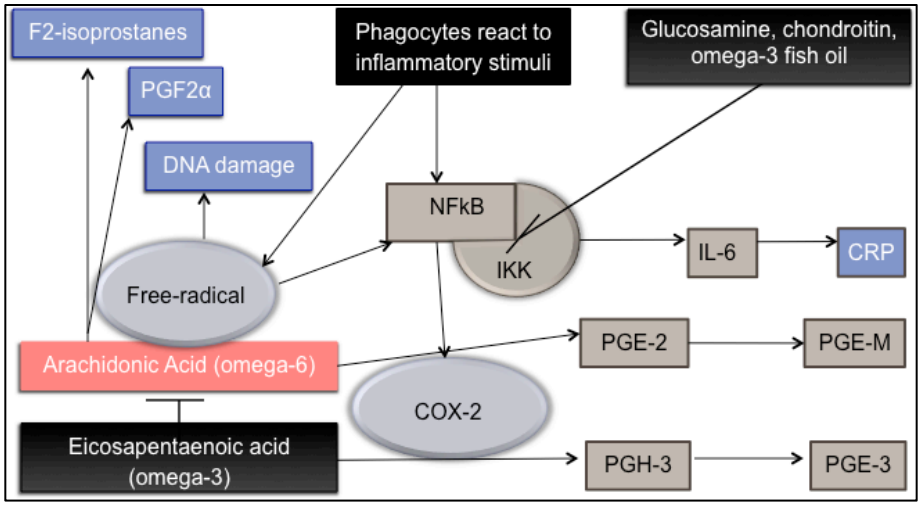
Colorectal cancer (CRC) is the third most common cancer among men and women in the United States (1) and it is therefore important that we identify potential preventive agents. In an exploratory analysis conducted within the VITamins and Lifestyle (VITAL) cohort study, we previously observed that use of glucosamine, chondroitin, and omega-3 polyunsaturated fatty acid (omega-3 PUFA)-containing fish oil supplements was associated with a reduced risk of CRC (2). No prior studies have assessed the association between glucosamine and chondroitin use and CRC, and while a number of studies have been conducted on the associations between CRC and dietary omega-3 PUFA intake and fish consumption, results have been mixed (3-6). Importantly, no prior studies have reported on the association between fish oil supplement use and CRC and few have considered total exposure to omega-3 PUFA (from diet and supplements) in relation to risk of CRC.

Given the limited research in this area and the need to identify safe, effective, and easily implemented preventives, it is essential to better understand the associations between use of glucosamine, chondroitin, and fish oil supplements and CRC, as well as the biologic mechanisms by which these supplements may reduce the risk of CRC. Inflammation and oxidative stress/DNA damage offer two potential mechanisms by which these supplements may reduce the risk of CRC.

Inflammation has been implicated in the etiology of CRC (7-11) and research suggests that glucosamine and chondroitin supplements have anti-inflammatory properties. *In vitro* studies have shown that glucosamine and chondroitin inhibit activity of nuclear factor kappa B (NFkB) (12, 13), a transcription factor central to the inflammatory cascade (14) (Figure 1). Corroborating *in vitro* and animal studies have demonstrated that administration of glucosamine and chondroitin reduces markers of inflammation downstream of NFkB (12, 15-18). In spite of this growing body of laboratory evidence, only two small human studies have been conducted on the association between glucosamine and chondroitin and inflammation. In a small study conducted among rheumatoid arthritis patients,

administration of glucosamine for 3 months did not affect inflammation as measured C-reactive protein (CRP) (19), though it should be noted that persons with rheumatoid arthritis have higher levels of inflammation than the general population, limiting the generalizability of these findings. In a second study conducted among persons with osteoarthritis, Nakamura et al. observed that 3 months of glucosamine+chondroitin administration significantly reduced inflammation, as measured by prostaglandin E_2 (PGE_2) concentration, among 36 persons with osteoarthritis (20).

Figure 1. Biologic pathways by which glucosamine, chondroitin, and omega-3 fish oil supplements may affect inflammation, oxidative stress, and DNA damage.



Exposures (glucosamine, chondroitin, omega-3 fish oil) shown in black; outcomes in blue

Omega-3 PUFAs contained within fish oil supplements have also been shown to have anti-inflammatory properties, with laboratory studies showing that omega-3 PUFAs reduce inflammation by inhibition of NFkB activity (21) and by competitive inhibition of pro-inflammatory omega-6 PUFAs, such as arachidonic acid (21-23). By competing with omega-6 PUFAs for storage in the cell membrane and for cyclooxygenase-2 (COX2) enzyme activity (Figure 1), omega-3 PUFAs act to competitively inhibit the pro-inflammatory effects of omega-6 PUFAs. By inhibiting omega-6 PUFAs, omega-3 PUFAs give rise to the less-inflammatory prostaglandin, PGE_3, in place of the strongly pro-inflammatory omega-6 derived prostaglandin, PGE_2. The anti-inflammatory effect of fish oil has been shown to extend to humans, with two recent randomized control trials showing that fish oil reduces circulating levels of inflammatory biomarkers (24, 25).

Even though inflammation is closely related to oxidative stress, less research has been conducted on the association between glucosamine, chondroitin, and fish oil supplements and oxidative stress. Laboratory research suggests that glucosamine (26, 27) and chondroitin (28, 29) supplements may act to reduce oxidative stress, though these associations have not been assessed in humans. More work has been conducted on the association between fish oil and oxidative stress/DNA damage, though the effect of fish oil on oxidative stress and DNA damage remains unclear (30-33). It is of interest to examine the association of these supplements and oxidative stress/DNA damage in humans, as oxidative stress and ensuing DNA damage have been suggested to play a role in several cancers, including CRC, though evidence from prospective human studies remains limited (34-41). Oxidative stress results from an imbalance of reactive species and anti-oxidant defenses, in which excess reactive species, or free radicals, damage cellular components, including lipid membranes and DNA (42-44) (Figure 1). Inflammation can contribute to this imbalance, as phagocytes release reactive species in response to inflammatory stimuli. However, reactive species themselves can also initiate NFkB activity, affecting downstream cytokine production. It is therefore difficult to tease apart these closely-related pathways. Examining associations between glucosamine, chondroitin, and fish oil use in relation to inflammation and oxidative stress may offer insight to the mechanisms by which these supplements may act to reduce risk of cancer.

In this study, we have sought to better understand the association between glucosamine, chondroitin, and fish oil supplements and CRC. With an additional 2 years of follow-up in the VITAL prospective cohort, we have the power to examine these associations in more detail and have assessed whether these associations follow a dose-response association, and have further evaluated whether these associations vary by formulation/source, subsite, and subgroup (factors associated with inflammation and/or underlying risk). We have also examined whether use of these supplements is associated with oxidative stress [urinary 8-isoprostane and prostaglandin 2-alpha (PGF2α)] and DNA damage (baseline

DNA damage, DNA repair capacity at 15 and 60 minutes) in a study of 220 persons included in the VITAL biomarker study. Furthermore, within a population of 9,947 adults aged 25+ included in the National Health And Nutrition Examination Survey (NHANES), we have tested whether use of glucosamine, chondroitin, and fish oil is associated with inflammation, as measured by plasma high-sensitivity C-reactive protein (hsCRP).

Glucosamine, chondroitin, and fish oil are among the most commonly used supplements in the United States making it critical to better understand any beneficial effects they provide. If use of glucosamine, chondroitin, or fish oil is, in fact, associated with decreased systemic inflammation and/or oxidative stress in humans, this may provide biologic evidence to substantiate the epidemiologic association observed between use of these supplements and reduced risk of CRC. Further study of these epidemiologic associations is an important step to exploring the chemopreventive potential of these supplements and may pave the way to future research. CRC is a major cause of morbidity and mortality in the United States making it important that we identify potential preventive agents that are safe, effective, and easily implemented.

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Chapter 1: Use of glucosamine and chondroitin supplements and risk of colorectal cancer

ABSTRACT

Glucosamine and chondroitin are non-vitamin, non-mineral supplements which have anti-inflammatory properties. These supplements are typically used for joint pain and osteoarthritis, and are commonly taken as either glucosamine alone or glucosamine plus chondroitin. An exploratory analysis conducted within the VITamins And Lifestyle (VITAL) study observed any use of glucosamine and chondroitin to be associated with reduced risk of colorectal cancer (CRC). With two additional years of follow-up, we have studied these associations in greater depth, including associations by frequency/duration of use and by formulation, and have evaluated whether observed associations are modified by factors associated with inflammation. Participants include 75,137 western Washington residents aged 50-76 who completed the mailed VITAL questionnaire between 2000- 2002. Use of glucosamine and chondroitin was ascertained by questions about supplement use during the 10-year period prior to baseline, and participants were followed for CRC through 2008 (n=557). Cox regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). Persons reporting use of glucosamine+chondroitin on 4+ days/week for 3+ years had 45% lower CRC risk than non-users (HR:0.55; 95% CI:0.30-1.01; p-trend:0.16). This association varied by body mass index (p-interaction: 0.006), with inverse association observed among the overweight/obese (p-trend: 0.02), but not among the underweight/normal-weight. Use of glucosamine alone was not significantly associated with CRC risk. There is great need to identify safe and effective cancer preventive strategies, and results suggest that glucosamine and chondroitin may merit further attention as a potential chemopreventive agent.

INTRODUCTION

Glucosamine and chondroitin are non-vitamin, non-mineral specialty supplements commonly used for joint pain and osteoarthritis. These supplements are often but not always taken together in a single daily supplement, and are sometimes additionally coupled with methylsulfonylmethane (MSM). While the effectiveness of these supplements on joint pain and function is debated (1-5), glucosamine and chondroitin are among the most commonly used supplements in the United States: 7.4% of older adults report use of glucosamine-chondroitin, a prevalence of use comparable to acetaminophen (6).

Beyond the inconclusive randomized control trial evidence for an effect on joint function (3-5), results of human, animal, and laboratory studies suggest that glucosamine and chondroitin may have anti-inflammatory properties (7-13). Given that chronic inflammation has been linked to the development of colorectal cancer (CRC) (14-19), there is substantial interest in assessing whether factors which reduce inflammation have potential utility in CRC prevention. To this end, an exploratory analysis of 11 supplements conducted within the VITamins And Lifestyle (VITAL) study observed that use of glucosamine and chondroitin supplements were associated with reduced risk of CRC (20).

With an additional 2 years of follow-up in the VITAL cohort, we have further investigated the associations between use of glucosamine and chondroitin supplements and CRC, with analyses designed to examine associations by formulation and by dose-response. We also assessed whether observed associations were modified by factors associated with inflammation and whether associations varied by cancer subsite and stage.

METHODS

Study Population

Study participants were drawn from the VITAL study, a prospective cohort of persons aged 50-76 years residing in the 13-county western Washington catchment area of the Surveillance, Epidemiology, and End Results (SEER) cancer registry (21). Potential participants were identified by purchased commercial mailing list, and were mailed a 24-page questionnaire and reminder postcard between October 2000 and November 2002. Of the 364,418 persons included in mailings, 77,719 returned the questionnaire and met eligibility requirements. We excluded persons with a history of CRC as of baseline (n=971), as well as those for whom this information was missing (n=213). We also excluded persons with history of ulcerative colitis or Crohn's disease (n=1030), intestinal polyposis (n=273), or malabsorptive syndromes (n=42). Additional exclusion criteria included diagnosis with *in situ* CRC over follow-up (n=12), cancer noted on death certificate only with no diagnosis date available (n=1), and diagnosis with CRC of certain rare morphologies, including malignant carcinoid tumors and lymphomas (n=33). Persons were also excluded if missing information on use of glucosamine, chondroitin, and MSM supplements (n=60), leaving 75,137 persons for analyses. The above-listed exclusions are not mutually exclusive and persons may have been excluded for more than one reason.

Exposure

Use of glucosamine, chondroitin, and MSM supplements was ascertained by a series of questions about use of various supplements in the 10-year period prior to baseline, including years of use and average number of days/week of use. For all analyses, our reference group was defined as non-users of glucosamine, chondroitin, and MSM supplements so as to yield the most pure group for comparison.

We classified use of glucosamine into 3 categories: high use (4+ days/week for 3+ years), low use (<4 days/week or <3 years), or no use of glucosamine, chondroitin, or MSM. These categories were created

a priori so that the highest level of exposure is defined by high frequency (4+days/week) and substantial duration (3+ years) of use. Given that approximately 72% of glucosamine users also report use of chondroitin or MSM, we created a second glucosamine variable in order to parse apart effects of these supplements and examine the effects of glucosamine alone. For this variable, users were defined as persons using glucosamine only (persons reporting chondroitin or MSM were excluded from these analyses).

We were unable to examine of the association between chondroitin and CRC independently, as over 99% of chondroitin users in our study also reported use of glucosamine or MSM. We have therefore created a variable to capture joint glucosamine+chondroitin use, with high use defined by use of both glucosamine and chondroitin supplements on 4+ days/week for 3+ years. Persons were classified as low use if they reported use of glucosamine and chondroitin but did not meet the definition of high use for both supplements, while non-use was defined as non-use of glucosamine, chondroitin, and MSM. Persons reporting use of either glucosamine or chondroitin alone were excluded from these analyses.

MSM was defined as high use (4+ days/week for 3+ years), low use (<4 days/week or <3 years), or no use of glucosamine, chondroitin, or MSM. We were unable to create an "MSM alone" variable, as this less-commonly used supplement is rarely taken in the absence of glucosamine and/or chondroitin: 83% of MSM users also report use of glucosamine or chondroitin.

Potential confounders

Covariates included in multivariate analyses were selected *a priori* and include factors associated with CRC, as well as factors associated with glucosamine use, including: older age, female gender, increased levels of physical activity, never smoking, and history of osteoarthritis or joint pain (22). Our multivariate analyses therefore include the following covariates: age (time-metric of analysis), sex, race/ethnicity (white, Hispanic, black, American Indian/Alaska Native, Asian or Pacific Islander, or other), educational

status (high school graduate/GED or less, some college or technical school, or college graduate or above), body mass index (BMI)(kg/m^2; classified as normal weight[<25], overweight[25-<30], obese[30-<35], and severely obese[35+]), physical activity (no moderate/vigorous activity, sex-specific tertiles of MET-hours per week of moderate/vigorous physical activity), smoking history (never, quit 10+ years before baseline, quit <10 years before baseline, current), energy intake (quartiles), total calcium intake (quartiles of dietary+supplemental intake), alcohol consumption (none-<1 drink/mo, 1 drink/mo-<4 drinks/wk, >4 drinks/wk-<2 drinks/day, 2+ drinks/day), multivitamin use (never, past, current), dietary fiber intake (quartiles), fruit/vegetable intake excluding potatoes (quartiles), red/processed meat intake (quartiles), hormone replacement therapy (never, former, current), as well as aspirin use and non-aspirin NSAID use (none, low, high use; high use defined by use 4+ days/week for 4+ years). Analyses also included adjustment for family history of CRC among 1^{st} degree relatives (yes/no), history of sigmoidoscopy/ colonoscopy in the 10 years prior to baseline (yes/no), and history of polyp excision (yes/no). We also adjusted for history of osteoarthritis or joint pain, as these conditions are the usual indications for use of glucosamine, chondroitin or MSM supplements.

BMI was determined by self-reported height and baseline weight. 1323 persons were missing baseline weight, but reported weight at age 45. For this group, we estimated baseline BMI by first calculating the average BMI change/year within sex-age-race group among those with complete data. We then applied the group-specific average BMI change/year to the number of years elapsing since age 45.

Information on diet was ascertained by a food-frequency questionnaire (FFQ) adapted from the Women's Health Initiative (23) which captures frequency and serving size of 120 foods and beverages consumed over the year prior to baseline. Participants were excluded from nutrient calculations if they did not complete all pages of the FFQ, or if they reported abnormally high or low energy intake (men: <800 kcal/day or >5000 kcal/day; women: <600 kcal/day or >4000 kcal/day).

Outcome

Cases were identified by linkage to the western Washington SEER registry, which uses information from area hospitals, state death certificates, as well as offices of oncologists, radiologists, and pathologists to find cases. Between baseline and December 2008, 557 invasive cancers of the colon and rectum were diagnosed.

Statistical Analysis

Cox regression was used to estimate hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) for associations between supplement use and CRC risk, with exposures and covariates modeled using indicator variables. For exposures under study, we additionally present tests for trend, with corresponding p-values obtained by alternatively modeling the exposures as continuous categorical variables. Analyses were conducted using Stata (version 12, College Station, TX). Study participants provided informed consent and study procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board.

Participants entered analysis at the time baseline questionnaire was received and were followed for an average of 6.7 years. Cases were followed until date of CRC diagnosis and non-cases were censored at whichever occurred earliest: date of death (6.7%), date of emigration out of the SEER catchment area (5.5%), date of requested removal from study (0.03%), or December 31st, 2008 (87.8%). Deaths occurring within the state of Washington were identified by linkage to the state death file, while emigrations out of area were identified by linkage to the National Change of Address System and by telephone calls and mailings.

We additionally tested for effect modification of the association between glucosamine+chondroitin use and CRC by factors associated with inflammation, including: gender, regular aspirin use in the 10-years prior to baseline (yes/no; regular use defined as use on 1+ days/week for 1+years), and BMI (<25, 25+).

A single interaction term was used to test for interaction, with glucosamine+chondroitin modeled as a continuous-categorical variable. We also tested for differences in association across cancer site (colon vs. rectum) and stage (local vs. regional/distant). In determining the HRs associated with each particular subsite, cases of the subsite not under study were censored at date of diagnosis. For analyses of stage, we instead opted to exclude cases of the stage not under study, given concern that censoring of these cases would violate the assumption of non-informative censoring. Logistic regression limited to cases was used to determine the statistical significance of subsite-and stage-specific differences.

RESULTS

Persons in the overall cohort reported a mean age of 61.4 years at baseline, and cases reported a mean age of 65.8 years. Cases reported drinking more alcoholic beverages than the overall cohort and also reported engaging in less physical activity (Table 1). As compared to the overall cohort, cases were also less likely to have had sigmoidoscopy/colonoscopy in the 10 years prior to baseline.

Persons reporting high use of glucosamine (4+ days/week for 3+ years in the 10 year period prior to baseline), with or without use of chondroitin or MSM (n=5,395), had a 29% lower risk of CRC as compared to persons reporting no use of glucosamine, chondroitin, or MSM in this time frame (HR: 0.71; 95% CI: 0.46, 1.11; p-trend: 0.19) (Table 2). When the definition of glucosamine use was limited to use of glucosamine alone (in which persons reporting any use of chondroitin or MSM were excluded from analyses), high use of glucosamine (n=1,229) was less strongly associated with CRC risk (HR: 0.86; 95% CI: 0.38, 1.94; p-trend:0.84). Persons reporting high use of glucosamine+chondroitin (n=3,481) had 45% lower risk of CRC than persons not using glucosamine, chondroitin, or MSM in the 10 years prior to baseline (HR: 0.55; 95% CI: 0.30, 1.01), though the test for trend did not reach significance (p-trend: 0.16). High use of MSM, which is usually, but not always, taken as part of a glucosamine and chondroitin supplement, was associated with a 52% reduced risk of CRC as compared to no use of glucosamine, chondroitin, or MSM (HR: 0.48; 95% CI: 0.12, 1.94; p-trend: 0.14).

We found no evidence for effect modification by gender (p-interaction: 0.19) or aspirin use (p-interaction: 0.19) (Table 3). However, we did observe that the association between glucosamine+chondroitin and CRC varied by BMI (p-interaction: 0.006), with no association observed among those of normal weight and an inverse association observed among the overweight/obese (p-trend: 0.02). Among the overweight/obese group, persons reporting high use of glucosamine+chondroitin experienced 72% lower risk of CRC than non-users of glucosamine,

chondroitin, and MSM (HR: 0.28; 95% CI: 0.10, 0.76). The association between glucosamine+chondroitin use and cancer risk did not vary by subsite (p-difference: 0.56) or stage (p-difference: 0.19) (Table 3).

DISCUSSION

In this prospective cohort study, use of glucosamine with or without chondroitin for 4+ days/week for 3+years was associated with a non-significant 29% reduction in CRC risk (HR: 0.71; 95% CI: 0.46-1.11; p-trend: 0.19). The association was stronger for the use of the combination glucosamine+chondroitin; those who used the combination for 4+ days/week for 3+ years had 45% lower risk of CRC as compared to non-users of glucosamine, chondroitin and MSM (HR: 0.55; 95% CI: 0.30, 1.01) though the overall trend did not reach statistical significance (p-trend: 0.16). The association between glucosamine+chondroitin and CRC varied by BMI (p-interaction: 0.006), with the risk reduction limited to those who were overweight or obese. Use of glucosamine alone was not associated with CRC risk.

In the prior exploratory analysis conducted within the VITAL cohort, any use of glucosamine was associated with a 27% reduced risk (HR: 0.73; 95% CI: 0.54-0.98; p: 0.03) and any use of chondroitin in the 10-year period prior to baseline was associated with a 35% reduced risk of CRC (HR: 0.65; 95% CI: 0.45-0.93; p: 0.02) (20). With an additional two years of follow-up in our current study, we similarly observed reduction in CRC risk associated with use of these supplements, though results did not reach statistical significance. If patterns of use changed after baseline and the etiologically relevant time frame for cases developing later in follow-up extends into follow-up, then we might expect a change (and therefore misclassification) of exposure status to attenuate results toward the null, possibly explaining why our results were somewhat weaker than those reported in the initial exploratory analysis. In our current study, we further explored associations between these supplements and CRC by formulation. Given that results were stronger for glucosamine+chondroitin (p-trend: 0.16) than for glucosamine alone (p-trend: 0.84), it seems possible that either chondroitin or the combination of glucosamine+chondroitin may be driving our observed effects. However, such conclusions are tempered by the lack of statistical significance of our associations, and by the fact that we were unable to examine

the association between chondroitin alone and CRC since chondroitin is rarely taken without glucosamine.

Beyond the prior VITAL study, no other studies have reported on the association between glucosamine and chondroitin supplements and CRC risk. However, results of our study may be compared to studies of aspirin, another anti-inflammatory which has been extensively studied in terms of CRC risk. A recent meta-analysis of randomized control trials (RCTs) has shown that aspirin use reduces risk of CRC by 24% (HR: 0.76, 95% CI: 0.60-0.96) (24), with the association strengthening when restricted to persons allocated to 5 or more years of use (HR: 0.68; 95% CI: 0.54-0.87) (24). Research also suggests that frequency of use may be an important component of the association between aspirin and CRC. In both the Nurses' Health Study (25) and Health Professionals Follow-up Study (26), Chan and colleagues observed increasing frequency of aspirin use to be associated with decreasing risk of CRC (p-trend: 0.007, 0.004, respectively). To this end, we have incorporated both duration (years) and frequency of use (days/week) into our supplement variables. In our study, we see that the trend for 'dose' is not significant for any of the supplements under study, with strongest associations observed among the high 'dose' users. It is possible that we did not observe a significant trend as a combination of high frequency and long duration of use may be needed to see effect. It is also possible that our power to detect significant association was limited by the small number of supplement users.

In addition to being associated with CRC risk, use of glucosamine and chondroitin supplements has been associated with reduced risk of lung cancer (20,27) and total mortality (22,28) in VITAL. In particular, Brasky et al. observed use of glucosamine (with or without chondroitin) on 4+days/week for 3+years to be associated with a 51% reduced risk of adenocarcinoma of the lung (HR:0.49; 95% CI:0.27-0.90) (27), a finding which aligns with randomized trial evidence showing that aspirin reduces risk of lung adenocarcinoma (29). It should also be noted, however, that other studies conducted within VITAL have

not observed association between glucosamine/chondroitin use and bladder cancer (30), prostate cancer (31), breast cancer (32), or hematologic cancers (33).

In our study, we observed that the association between glucosamine+chondroitin use and CRC risk varied by BMI (p-interaction: 0.006), with significant inverse association observed among those who are overweight/obese (p-trend: 0.02), and no association observed among the underweight/normal-weight. This interaction supports our hypothesis that these posited anti-inflammatory supplements may be most strongly associated with CRC risk among the group likely to have highest inflammation, (i.e., the overweight/obese) (34). While this question has not been previously addressed in terms of glucosamine and chondroitin, studies have assessed whether BMI modifies the association between aspirin/NSAID use and CRC, and results have been inconsistent. A recent RCT reported that normal-weight persons and overweight persons given 325 mg aspirin/day experienced no reduction in risk of advanced adenoma as compared to persons receiving the placebo (RR: 1.23; 95% CI: 0.55-2.77; RR: 0.94; 95% CI: 0.54-1.64, respectively), while a RR of 0.44 (95% CI: 0.17-1.10) was reported among the obese (35). However, another study conducted within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) suggested just the opposite: an inverse association was observed between NSAID use and risk of left-sided adenoma among women with BMI<25, while no association was observed among women of higher BMI (36).

If these supplements have the potential to reduce risk of CRC, it would likely be through reduction in inflammation. Laboratory research suggests that glucosamine and chondroitin may affect inflammation through the nuclear factor kappa B (NF-κB) pathway (10-12). NF-κB is a transcription factor which lies upstream of many inflammatory processes and which has been implicated in various inflammation-related cancers (37). Recent research conducted using a mouse epidermal cell line demonstrated that chondroitin sulfate inhibits NF-κB activity by preventing degradation of the NF-κB inhibitory subunit, IκB

(11). Glucosamine has been shown to similarly inhibit NF-κB activity in a dose-dependent manner (10). In support of these findings, *in vitro* and animal studies suggest that glucosamine and chondroitin administration is associated with decreased levels of various inflammatory biomarkers associated with the NF-κB pathway, including IL-1β, IL-6, PGE_2, and TNF-α (7-10,38). *In vivo* research conducted in rats with chemically-induced colitis has further demonstrated that glucosamine reduces colonic inflammation and that both glucosamine and chondroitin have therapeutic effects, potentially reflecting the possible anti-inflammatory effects of these supplements (39,40).

Two studies suggest that these anti-inflammatory effects may extend to humans. Nakamura and colleagues measured serum PGE_2 in a group of 36 persons with osteoarthritis at baseline and after 3 months of glucosamine-chondroitin administration (9). Study authors observed a significant decrease in serum PGE_2, and the post-intervention PGE_2 level among the osteoarthritic group was similar to the serum PGE_2 level among 25 age-matched healthy controls. This evidence, while limited, is corroborated by a large recent observational study conducted within the National Health And Nutrition Examination Survey (NHANES), a nationally-representative sample of nearly 10,000 US adults, in which we observed that persons reporting regular current chondroitin use had 22% lower levels of circulating serum CRP than non-users (95% CI: 8%-33%), and current glucosamine users had 17% lower CRP than non-users (95% CI: 7%-26%) (13).

Our study may be limited by measurement error in supplement use, as supplement use was ascertained by self-report. While we have information on the days/week and years of use, we do not have information on the dose used, preventing estimation of average daily dose. Another potential limitation of this study is residual confounding. This may be of particular concern when studying healthy behaviors, such as supplement use. To address this concern, we have adjusted for several potential confounding factors, including factors associated with health behaviors (i.e., history of sigmoidoscopy/colonoscopy,

use of multivitamins, education, and smoking history, among others). We are also limited by small sample size especially when conducting subgroup analyses, limiting our ability to detect subgroup-specific associations.

Our study also has several strengths. Since VITAL was designed to assess the association between supplement use and cancer, we have information on supplement use in the 10-year period prior to baseline, as well as detailed information on various potential confounding factors, including several health behaviors and conditions that are indications for supplement use. It should also be noted that we have shown good reliability and validity of supplement use as reported in the VITAL baseline questionnaire (41), though the reliability and validity of these specific supplements have not been examined. Furthermore, we expect nearly complete case ascertainment, as cases were identified by annual linkage to SEER.

In summary, our research indicates that use of glucosamine plus chondroitin may merit further attention as a potential chemopreventive agent, though a larger study, with a larger number of supplement users, may be needed to assess these associations in greater detail. Since CRC is major cause of morbidity and mortality, it is important that we seek potential preventive strategies which are safe, effective, and easily implemented. Glucosamine and chondroitin supplements have been shown to be safe (4,42-44) and are already widely used (6), and it is therefore important that we seek to better understand the suggestive association between use of these supplements and CRC.

Table 1. Distribution of Colorectal Cancer Risk Factors among VITAL Cohort Participants and Cases

	Cohort (n=75,137) *n* (%)	Cases (n=557) *n (%)*
Age at Baseline (yrs)		
50- <55	17,590 (23.4)	50 (8.98)
55- <60	17,109 (22.8)	72 (12.9)
60- <65	13,720 (18.3)	87 (15.6)
65- <70	12,340 (16.4)	149 (26.8)
70+	14,378 (19.1)	199 (35.7)
Sex		
Female	38,981 (51.9)	274 (49.2)
Male	36,156 (48.1)	283 (50.8)
Body Mass Index (m/kg^2)		
Normal Weight (<25)	24,984 (34.3)	163 (30.5)
Overweight (≥25-<30)	29,955 (41.2)	208 (38.9)
Obese (≥30-<35)	12,023 (16.5)	109 (20.4)
Severely Obese (≥35)	5,805 (8.0)	55 (10.3)
Physical Activity (MET-hrs per week mod/vig activity)[a]		
None	38,344 (51.7)	336 (61.8)
Tertile 1	11,993 (16.2)	77 (14.2)
Tertile 2	11,849 (16.0)	67 (12.3)
Tertile 3	11,918 (16.1)	64 (11.8)
Alcoholic Drinks (drinks)		
None- <1/mo	27,502 (37.6)	202 (37.4)
1/mo -≤4/wk	21,720 (29.7)	141 (26.1)
>4/wk -<2/day	15,752 (21.5)	102 (18.9)
2+/day	8,275 (11.3)	95 (17.6)
Red/ Processed Meat Intake (oz/week)[b]		
Quartile 1: <9.11	17,065 (25.0)	104 (21.3)
Quartile 2: 9.11- <17.1	17,065 (25.0)	133 (27.2)
Quartile 3: 17.1-<28.3	17,065 (25.0)	111 (22.7)
Quartile 4: 28.3+	17,064 (25.0)	141 (28.8)
Dietary Fiber Intake (g/day)		
Quartile 1: <12.4	17,065 (25.0)	130 (26.6)
Quartile 2: 12.4- <17.4	17,065 (25.0)	140 (28.6)
Quartile 3: 17.4- <23.7	17,065 (25.0)	116 (23.7)
Quartile 4: 23.7+	17,064 (25.0)	103 (21.1)
Aspirin Use[c]		
Non-user	40,177 (55.1)	322 (59.9)
Low (<4 days per week or <4 years)	17,113 (23.5)	115 (21.4)
High (4+ days per week for 4+ years)	15,693 (21.5)	101 (18.8)
History of Sigmoidoscopy/Colonoscopy (last 10 yrs)		
No	32,681 (43.9)	290 (52.6)
Yes	41,816 (56.1)	261 (47.4)

[a] Tertiles of physical activity among those engaging in moderate/vigorous leisure time physical activity determined within gender; women (T1: <2.81; T2: 2.81-<9.47; T3: 9.47+); men (T1: <-4.34; T2: 4.34- <13.2; T3: 13.2+)

[b] 1 oz=28.35 g

[c] Use of aspirin (including both low-dose and regular aspirin) defined by use over 10 years prior to baseline

Table 2. Hazard Ratios (HR) of Colorectal Cancer Associated with Use of Glucosamine, Glucosamine Plus Chondroitin, and Methylsulfonylmethane (MSM) Supplements over the 10 Years Prior to Baseline

Supplement Use	Cohort N (%)	Cases N (%)	Age and Sex Adjusted HR	Age and Sex Adjusted 95% CI	Multivariate Adjusted [a] HR	Multivariate Adjusted [a] 95% CI
Glucosamine [b]						
No Use of Glucosamine, Chondroitin, MSM	59,024 (79.6)	461 (83.2)	1.00	Ref	1.00	Ref
Low Use (<4 days/week or <3 yrs)	9,724 (13.1)	63 (11.4)	0.79	0.61, 1.03	0.98	0.72,1.32
High Use (≥4 days/week and ≥3 years)	5,395 (7.28)	30 (5.42)	0.62	0.43,0.89	0.71	0.46,1.11
			P-trend: 0.003		*P-trend: 0.19*	
Glucosamine Alone [c]						
No Use of Glucosamine, Chondroitin, MSM	59,024 (93.3)	461 (94.5)	1.00	Ref	1.00	Ref
Low Use (<4 days/week or <3 years)	3,022 (4.78)	21 (4.30)	0.88	0.57,1.37	1.04	0.63,1.73
High Use (≥4 days/week and ≥3 years)	1,229 (1.94)	6 (1.23)	0.56	0.25,1.26	0.86	0.38,1.94
			P-trend: 0.15		*P-trend: 0.84*	
Glucosamine Plus Chondroitin [d]						
No Use of Glucosamine, Chondroitin, MSM	59,024 (85.5)	461 (88.0)	1.00	Ref	1.00	Ref
Low Use (<4 days/week or <3 years)	6,509 (9.43)	44 (8.40)	0.81	0.59,1.11	1.07	0.75,1.51
High Use (≥4 days/week and ≥3 years)	3,481 (5.04)	19 (3.63)	0.60	0.38.0.95	0.55	0.30,1.01
			P-trend: 0.01		*P-trend: 0.16*	
Methylsulfonylmethane (MSM) [e]						
No Use of Glucosamine, Chondroitin, MSM	59,024 (94.2)	461 (96.4)	1.00	Ref	1.00	Ref
Low Use (<4 days/week or <3 years)	2,852 (4.55)	12 (2.51)	0.51	0.29,0.91	0.72	0.39,1.32
High Use (≥4 days/week and ≥3 years)	753 (1.20)	5 (1.05)	0.79	0.33,1.90	0.48	0.12,1.94
			P-trend: 0.05		*P-trend: 0.14*	

Abbreviations: HR (hazard ratio); MSM (methylsulfonylmethane); 95% CI (95% confidence interval)

[a] Multivariate model adjusted for age, sex, race/ethnicity, education, BMI, energy intake, MET-hours per week of moderate/vigorous activity, alcohol intake, smoking history, multivitamin use, calcium intake (diet+supplement), dietary fiber intake, fruit and vegetable intake (excluding potatoes), red/processed meat intake, aspirin use, non-aspirin NSAID use, family history of colorectal cancer, history of sigmoidoscopy/colonoscopy, history of polyp excision, hormone replacement therapy, and history of arthritis or joint pain

[b] High/low use of glucosamine defined by use of glucosamine, regardless of whether participant also used chondroitin and/or MSM

[c] High/low use of glucosamine alone defined by use of glucosamine only; participants using glucosamine in addition to chondroitin and/or MSM are excluded from analyses

[d] Users of glucosamine alone or chondroitin alone excluded from these analyses

[e] 83% of users of MSM are also users of glucosamine or chondroitin.

Table 3. Hazard Ratios (HR) of Colorectal Cancer Associated with Use of Glucosamine Plus Chondroitin, by Gender, Aspirin Use, Body Mass Index, Cancer Subsite, and Stage

	10-yr Use of Glucosamine Plus Chondroitin Supplements										
	No Use			Low Use (<4 days/week or <3 yrs)			High Use (≥4 days/week and ≥3 yrs)				
	Case/ Cohort	HR[b]	95% CI	Case/ Cohort	HR[b]	95% CI	Case/ Cohort	HR[b]	95% CI	P-trend	P-interaction[c]/ P-difference[d]
Gender											
Male	209/25,114	1.00	Ref	11/2,075	0.71	0.38,1.32	5/1,234	0.51	0.21,1.26	0.08	0.19
Female	136/21,618	1.00	Ref	27/3,306	1.41	0.91,2.17	6/1,606	0.63	0.27,1.46	0.94	
Aspirin Use[e]											
Not regular	208/26,194	1.00	Ref	26/2,631	1.44	0.94,2.22	6/1,288	0.57	0.25,1.30	0.78	0.19
Regular	137/20,538	1.00	Ref	12/2,750	0.68	0.37,1.25	5/1,552	0.50	0.20,1.24	0.07	
Body Mass Index											
<25 kg/m^2	96/15,831	1.00	Ref	14/1,840	1.49	0.83,2.68	7/948	1.14	0.51,2.52	0.40	0.006
≥25 kg/m^2	249/30,901	1.00	Ref	24/3,541	0.92	0.60,1.43	4/1,892	0.28	0.10,0.76	0.02	
Subsite[f]											
Colon	256/46,732	1.00	Ref	28/5,381	1.02	0.68,1.53	9/2,840	0.57	0.29,1.13	0.20	0.56
Rectum	89/46,732	1.00	Ref	10/5,381	1.24	0.63,2.45	2/2,840	0.45	0.11,1.85	0.54	
Stage[g]											
Local	160/46,547	1.00	Ref	14/5,357	0.84	0.48,1.48	4/2,833	0.42	0.15,1.16	0.09	0.19
Regional/Distant	181/46,568	1.00	Ref	23/5,366	1.23	0.78,1.93	7/2,836	0.66	0.31,1.44	0.68	

Abbreviations: HR (hazard ratio); 95% CI (95% confidence interval)

[a] Users of glucosamine alone or chondroitin alone excluded from these analyses

[b] Multivariate model adjusted for age, sex, race/ethnicity, education, BMI, energy intake, MET-hours per week of moderate/vigorous activity, alcohol intake, smoking history, multivitamin use, calcium intake (diet+supplement), dietary fiber intake, fruit and vegetable intake (excluding potatoes), red/processed meat intake, aspirin use, non-aspirin NSAID use, family history of colorectal cancer, history of sigmoidoscopy/colonoscopy, history of polyps, hormone replacement therapy, and history of arthritis or joint pain

[c] P-interaction used to test for effect modification by gender, aspirin use, and body mass index

[d] P-difference used to test for differences across cancer subsite and stage

[e] Regular use of aspirin (including both low-dose and regular aspirin) defined as use at least once a week for a year over the 10 year period prior to baseline

[f] Cancers of the subsite not under study included in subsite-stratified analyses and censored at date of diagnosis

[g] Cancers of stages not under study excluded from stage-stratified analyses

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Chapter 2: Long-chain omega-3 polyunsaturated fatty acid intake and risk of colorectal cancer

ABSTRACT

Research suggests that long-chain omega-3 polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-neoplastic properties, yet evidence for association between LC-PUFAs and colorectal cancer (CRC) remains inconsistent. Using the VITamins And Lifestyle (VITAL) cohort, we evaluated how EPA/DHA intake, and its primary sources, fish oil supplement use and dark fish consumption, relate to CRC risk. A total of 68,109 Washington residents aged 50-76 completed a questionnaire between 2000-2002 and were followed for CRC through 2008 (n=488). Persons using fish oil supplements on 4+days/week for 3+years experienced 49% lower CRC risk than non-users (95% CI:0%,74%; p-trend: 0.06). The association between fish oil use and decreased CRC risk was primarily observed for men (p-interaction: 0.02; p-trend men: 0.02; p-trend women: 0.88) and for colon cancer (p-difference: 0.05; p-trend colon: 0.03; p-trend rectum: 0.87). While dark fish and total EPA+DHA intake were not associated with CRC risk overall, these associations varied by genetic risk (p-interaction: 0.009 and 0.02, respectively), with inverse associations observed among low-moderate genetic risk groups and positive associations observed among high risk groups. Results suggest that associations between LC omega-3 PUFA intake and CRC may vary by gender, subsite, and genetic risk, providing additional insight into the potential role of LC-PUFAs in cancer prevention.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer among men and women in the United States (1), and it is therefore important that we identify potential preventive agents. Inflammation has been implicated in the etiology of several cancers, including CRC, and has gained recent attention as a target for preventive efforts (2, 3).

Recent RCT evidence has demonstrated that long-chain omega-3 polyunsaturated fatty acids (PUFAs) eicosapentanenoic acid (EPA) and docosahexaenoic acid (DHA) reduce inflammation in humans (4, 5). These long-chain omega-3 PUFAs are found in dark fish and fish oil supplements, and may have additional anti-neoplastic properties, including anti-proliferative, pro-apoptotic, and anti-angiogenic effects (6-9).

Despite support for several anticancer mechanisms, observational data on the association between total long-chain omega-3 intake and CRC risk is inconsistent. While two meta-analyses have concluded that fish intake is associated with decreased risk of CRC (10, 11), two systematic reviews of omega-3 PUFAs on cancer risk qualitatively concluded that there is inadequate (12) or limited (13) evidence to suggest an association between long-chain omega-3 PUFA intake and CRC risk. Despite the number of studies conducted on dietary long-chain omega-3 PUFA intake and CRC, few studies have assessed the association between fish oil supplement use or total (diet+supplemental) long-chain omega-3 intake and CRC risk. This is important given the popularity of fish oil supplements and the fact that these supplements contain high doses of EPA and DHA, allowing for a broader range of exposure potentially not observable when considering dietary exposure alone.

In this paper, we follow up on a previous finding from the VITamins And Lifestyle (VITAL) study which found that any use fish oil supplements in the 10 years before baseline was associated with a reduced risk of CRC (14). With additional follow-up, we examine whether there is a dose-response association

between CRC and fish oil supplement use, and further evaluate whether CRC is associated with dark fish intake, dietary and total (diet+supplement) intake of EPA, DHA, EPA+DHA, and the omega-3 to omega-6 ratio. We also assess whether associations vary by anatomic subsite and by gender, body mass index (BMI), aspirin use, dietary fiber intake, history of polyps, and genetic risk.

METHODS

Study Population

The study population includes participants of the VITAL Study, a prospective cohort of persons aged 50-76 years residing in the 13-county Western Washington catchment area of the Surveillance, Epidemiology, and End Results (SEER) cancer registry (15). Between October 2000 and November 2002, 364,418 potential participants identified by purchased commercial mailing list were mailed a 24-page questionnaire and reminder postcard. 77,719 persons returned the questionnaire and were deemed eligible for inclusion in the VITAL cohort. Study procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board.

We excluded persons with a history of CRC as of baseline (n=971), as well as those for whom this information was missing (n=213). Persons with history or unknown history of the following conditions were also excluded: ulcerative colitis or Crohn's disease (n=1030), intestinal polyposis (n=273), and malabsorptive syndromes (n=42). Additional exclusion criteria included diagnosis with *in situ* CRC over follow-up (n=12), cancer noted on death certificate only (n=1), and diagnosis with CRC of certain rare morphologies (n=33). Persons missing information on diet (n=6,890) and fish oil supplement use (n=236) were further excluded, leaving 68,109 persons for analyses.

Exposure

Supplemental fish oil use was ascertained by a series of questions about use of fish oil/omega-3 supplements in the 10-year period prior to baseline, including years of use and the average number of days/week of use. From this information, we classified use into 3 categories (high use [4+ days/week for 3+ years], low use [<4 days/week or <3 years] or no use), with the high dose category including persons with both high frequency and duration of use.

Dark fish consumption and fatty acid intake were ascertained by a food-frequency questionnaire (FFQ) which captures frequency of intake and serving size of 120 foods and beverages (16). Participants were asked about their consumption of "dark fish such as salmon and fresh tuna" over the last year, from which we classified participants into quartiles of based on serving–size adjusted frequency. The University of Minnesota's Nutrition Coding Center Database was used to convert FFQ data into nutrient intake, including EPA and DHA intake. Participants were excluded from nutrient calculations if they did not complete all pages of the FFQ or if they reported abnormally high or low energy intake (men reporting <800 kcal/day or >5000 kcal/day; women reporting <600 kcal/day or >4000 kcal/day). For long-chain omega-3 intake, we present results in terms of i) dietary intake determined by the FFQ, and ii) total dietary+supplement intake. In calculating g/day of long-chain omega-3 PUFA intake from supplements, we calculated the average days/week in which fish oil supplements were used over the 10-year period prior to baseline. We then incorporated information on average EPA (0.64 g) and DHA (0.35 g) contained within popular fish oil supplements to calculate estimated average dose of supplemental EPA, DHA, and EPA+DHA intake over the 10-year period prior to baseline (17). Average daily intake from dietary and supplement sources was summed to estimate total intake (g/day), with results presented in quartiles. We also present quartiles of the omega-3 to omega-6 ratio, with omega-3 intake defined by total EPA+DHA intake and omega-6 intake including dietary arachidonic acid and linoleic acid.

Potential confounders

Age and sex are included in all models, while multivariate analyses include the following *a priori*-selected covariates: race/ethnicity (white, Hispanic, black, American Indian/Alaska Native, Asian or Pacific Islander, or other), educational status (high school graduate/GED or less, some college or technical school, or college graduate or above), BMI (kg/m^2; classified as normal weight[<25], overweight[25-<30], obese[30-<35], and severely obese[35+]), physical activity (no moderate/vigorous

activity, sex-specific tertiles of activity), smoking history (never, quit 10+ years before baseline, quit <10 years before baseline, current), energy intake (quartiles), total calcium intake (quartiles of dietary+supplemental intake), alcohol consumption (none-<1 drink/month, 1 drink/month-<4drinks/week, >4 drinks week/-<2 drinks/day, 2+ drinks/day), multivitamin use (never, past, current), dietary fiber intake (quartiles), fruit/vegetable intake (quartiles), red/processed meat intake (quartiles), omega-6 intake (quartiles), hormone replacement therapy (never, former, current), as well as aspirin use and non-aspirin non-steroidal anti-inflammatory drug (NSAID) use (none, low, high use; high use defined by use 4+ days/week for 4+ years). Analyses also included adjustment for family history of CRC among 1st degree relatives (yes/no), history of sigmoidoscopy/ colonoscopy in the 10 years prior to baseline (yes/no), and history of polyp excision (yes/no).

BMI was calculated from self-reported height and weight (kg/m^2) at baseline. For persons missing baseline BMI, but who reported height and weight at age 45 (n=1114), we estimated baseline BMI. This was achieved by first calculating the average BMI change/year within sex-age-race group among those with complete data, after which we applied the group-specific average BMI change/year to the number of years elapsing since age 45.

Physical activity (average MET-hours/week of moderate/vigorous activity) was ascertained by questions about activities in the 10-year period prior to baseline. Participants who reported engaging in a given activity regularly (at least 1 time/week for at least one year) in this 10-year period were asked to report on hours/day, days/week, and years of activity, plus intensity for walking. From this information, MET-hours/week of moderate/vigorous activity was calculated.

We also adjusted for conditions which may have increased fish oil supplement use or dark fish consumption, including: history of cardiovascular disease (coronary bypass surgery, angioplasty, angina, myocardial infarction, or stroke), hypercholesterolemia (use of cholesterol-lowering drugs), and memory

loss (memory better/same as age 25, memory somewhat worse than age 25, or memory much worse than age 25).

Outcome

Cases include 488 incident, invasive cancers of the colon and rectum diagnosed between baseline and December 31, 2008. Cases were identified by linkage to the western Washington SEER registry, which uses information from area hospitals, state death certificates, and offices of oncologists, radiologists, and pathologists to identify cases. VITAL is linked to SEER annually in a largely automated process, with datasets matched on data items common to both datasets, such as: name, Social Security number, and date of birth. If too few data items are in common to ensure a match, the datasets are reviewed manually, with participants telephoned directly if needed.

Effect modifiers/ Anatomic Subsite

We tested for effect modification and for difference across anatomic subsite (colon vs. rectum) for the following exposures: fish oil supplement use, dark fish consumption, and total long-chain omega-3 intake (EPA+DHA). Factors considered as potential effect modifiers include history of polyp excision (as this may represent a high-risk subgroup for chemoprevention) and factors associated with inflammation: gender, BMI (normal weight, overweight, obese), aspirin use (yes/no), and smoking status (never/quit >10 years prior to baseline vs current/quit within 10 years of baseline). Given recent evidence to suggest that dietary fiber may interact with long-chain omega-3 intake on CRC risk (18), we also tested for effect modification by dietary fiber intake (above/below median).

We also tested for effect modification by genetic risk score within a nested case-control study. Contributing cases and controls were selected in 2008 and DNA for genotyping was obtained from buccal swabs collected from cohort participants at the time of baseline questionnaire. Samples were processed at the Broad Institute using Illumina's HumanCytoSNP-12v2 beadchip, with samples excluded

in the genotyping process if they had low sample volume, low sample concentration, gender mismatch, or call rate <97%. Of those successfully genotyped, duplicates, non-whites and principal components analysis outliers have been further excluded, leaving 216 cases and 226 controls for analyses. An overall genetic risk score was created by enumerating the number of risk alleles present at 16 single nucleotide polymorphisms (SNPs) located within known/recently-identified CRC susceptibility loci including: rs6691170(1q41) rs6687758(1q41), rs10936599(3q26.2), rs16892766(8q23.3), rs6983267(8q24.21), rs10795668(10p14), rs3802842(11q23.1), rs11169552(12q13.13), rs7136702(12q13.13), rs4444235(14q22.2), rs4779584(15q13.3), rs9929218(16q22.1), rs4939827(18q21.1), rs10411210(19q13.1), rs961253(20p12.3), and rs4925386(20q13.33) (19, 20). The number of risk alleles present at each SNP was determined by either direct genotype or imputation as part of the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO). Two of these loci (1q41 and 12q13.13) include more than 1 SNP associated with CRC risk. We decided to include both SNPs at each of these loci in our risk score, as we did not find compelling evidence of correlation between these SNPs. In addition to the overall score, we also created an exploratory transforming growth factor-beta (TGF-β) risk score. This exploratory score included a subset of 6 SNPs (rs4444235, rs4779584, rs4939827, rs10411210, rs961253, rs4925386) included in the overall genetic risk score which have also been associated with the TGF-β pathway, a pathway important to various processes involved in carcinogenesis, including inflammation (19, 21, 22).

Statistical Analysis

Cox regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) between exposures of interest and CRC risk, with age used as the time metric of analysis. Exposures and covariates were modeled using indicator variables; where applicable, we also present tests for trend with corresponding p-values obtained by modeling exposures as continuous categorical variables. We tested for interaction using a single interaction term, with both exposure and effect modifier modeled

using “trend” variables. Participants entered analysis at the time the baseline questionnaire was received and cases were followed until date of CRC diagnosis, while non-cases were censored at whichever occurred earliest: date of death (6.7%), date of emigration out of the SEER catchment area (5.5%), date of requested removal from study (0.03%), or December 31st, 2008 (87.8%). Deaths occurring within the state of Washington were identified by linkage to the state death file, while emigrations out of area were identified by linkage to the National Change of Address System and by active follow-up involving telephone calls and mailings. Study participants have been followed for an average of 6.7 years.

In determining the subsite-specific HRs associated with colon cancer and rectal cancer, cases of the opposite subsite were censored at the date of diagnosis. To determine the statistical significance of subsite-specific differences, logistic regression was used to model the association between exposure and outcome (colon cancer vs rectal cancer; non-cases excluded), with the p-trend corresponding to the p-difference across subsite. For analyses involving the genetic risk score, we developed a reduced multivariate model by first deciding on a base model of covariates, after which we included additional covariates which changed the beta for interaction by >10%. Our final model for these analyses included age, sex, first 3 principal components to adjust for population substructure, energy intake, and alcohol consumption. All analyses were conducted using Stata (version 12, College Station, TX).

RESULTS

Analyses include 68,109 persons, among whom 488 cases of CRC occurred. In minimally-adjusted models presented in Table 1, increasing age and BMI were associated with increased risk of CRC. Risk of CRC declined with increasing educational status, physical activity, calcium intake, fiber intake, and fruit/vegetable intake. Recent smokers and current smokers were observed to be at increased risk of CRC, as were persons consuming >2 alcoholic drinks/week. High consumption of red/processed meat intake was also associated with increased CRC risk, while aspirin use, HRT, and history of sigmoidoscopy/colonoscopy were associated with decreased risk.

As compared to non-users, persons using fish oil supplements for 4+ days/week for 3+ years had reduced risk of CRC (HR: 0.51, 95% CI: 0.21, 1.00), though the test for trend was not significant (p-trend: 0.06) (Table 2). Dark fish consumption was not associated with CRC risk, nor was dietary or total (diet+supplemental) EPA, DHA or EPA+DHA intake. The omega-3 to omega-6 ratio was not associated with CRC risk.

We also conducted sensitivity analyses for associations between CRC and fish oil supplement use, dark fish consumption, and total omega-3 intake in which we excluded the first two years of follow-up. Exclusion of early follow-up strengthened results: the HR for high use of fish oil supplements strengthened to 0.38 (95% CI: 0.16, 0.93, p-trend:0.05), the HR for highest vs. lowest quartile of dark fish consumption was 0.66 (95% CI: 0.45, 0.99, p-trend:0.15), and the HR for the highest vs. lowest quartile of total EPA+DHA was 0.83 (95% CI: 0.58,1.20; p-trend:0.26).

In subsite-specific analyses, increasing fish oil supplement use was associated with reduced risk of colon cancer, but not rectal cancer (p for difference: 0.05): high supplement users experienced a 63% lower risk of colon cancer than non-users (95% CI: 9%-85%; p for trend 0.03) (Table 5). Neither dark fish intake

nor total EPA+DHA intake was associated with cancer at either site (Table 5), and no difference in association was observed between proximal and distal colon cancers (results not shown).

We observed significant interaction (p-interaction: 0.02) between fish oil supplement use and gender, with increasing dose associated with reduced CRC risk among men (p-trend: 0.02), but not women. As shown in Table 3, male high supplement users experienced 78% lower risk of CRC than non-users (95% CI: 10%, 94%). Furthermore, we observed significant interaction between dark fish consumption and overall genetic risk (p: 0.009) and between total EPA+DHA intake and overall genetic risk (p: 0.02) (Table 4). Increasing dark fish consumption and increasing total EPA+DHA intake were inversely associated with CRC risk among persons in the lowest two tertiles of genetic risk, while positive associations were observed among those in the highest tertile of genetic risk. When the genetic risk score was limited to 6 SNPs associated with the TGF-β pathway, no interaction was observed (results not shown). Given limited sample size of this nested case-control study, we were unable to assess interaction between genetic risk and dose of fish oil supplement use. No other interactions (BMI, aspirin use, dietary fiber intake, smoking status, history of polyp excision) were statistically significant (results not shown).

DISCUSSION

In this prospective study, persons using fish oil supplements for 4+ days/week for 3+ years had about half the risk of CRC of non-users, with the observed association driven by colon cancer rather than rectal cancer and by findings for men more than women. While dark fish consumption and long-chain omega-3 intake were not associated with CRC risk overall, these associations were significantly modified by genetic risk (p-interaction:0.009 and 0.02, respectively), with inverse associations observed in the low/moderate genetic risk groups and positive associations observed in the high genetic risk group.

Several epidemiologic studies have addressed the association between CRC and fish intake or dietary long-chain omega-3 PUFA intake. Two meta-analyses have reported significant inverse association between fish intake and CRC risk: a 2007 meta-analysis reported a relative risk (RR) of 0.88 (95% CI: 0.78, 1.00) comparing persons of the highest and lowest fish consumption categories (11), while a 2012 meta-analysis reported an odds ratio (OR) of 0.86 (95% CI: 0.79-0.95) (10). While our findings were not statistically significant, these point estimates are comparable in magnitude to the HR observed in our study for dark fish intake (HR: 0.77; 95% CI: 0.55, 1.07). Furthermore, it should be noted that in the 2012 meta-analysis referenced above, the association was stronger in case-control studies (OR: 0.83; 95% CI: 0.72-0.95) than in cohort studies (HR: 0.93, 95% CI: 0.86, 1.01) (10). A previous systematic review of prospective studies in 2006 concluded that there was no evidence to suggest an association between omega-3 PUFA intake and CRC risk (12), though overall point estimates were not presented. An updated systematic review of recently published studies (including both cohort and case-control studies) similarly concluded that there is only limited suggestion of an association between long-chain omega-3 PUFA intake and CRC risk (13), again not presenting an overall effect estimate. In part, these observed inconsistencies may reflect differences in exposure contrast across studies, as there is some suggestion that studies conducted within high exposure populations reveal stronger associations than studies with

less exposure contrast (23). Results from the 2006 meta-analysis by Geelen *et al* support this notion: a pooled RR of 0.95 was observed comparing highest to lowest fish consumption groups among 'low exposure contrast' studies in which the highest and lowest quartiles were separated by <7 fish eating occasions/month (95% CI: 0.81, 1.11), while a RR of 0.78 was observed among 'high exposure contrast' studies in which the highest and lowest quartiles were separated by 7+ fish eating occasions/month (95% CI: 0.66, 0.92) (11).

While our analyses of dietary exposures may have been influenced by insufficient exposure contrast, analysis of fish oil supplement use may allow for greater contrast, as these supplements contain high EPA and DHA levels. Persons using fish oil supplements on 4+days /week consume the approximate EPA+DHA as persons consuming 1.5 4-oz (112 g) servings salmon/week, a higher threshold of exposure than the upper quartile of dark fish consumption in our study (>0.8 servings/week). Here, we observed that persons who reported fish oil supplement use on 4+ days/week for 3+years experienced 49% lower CRC risk than non-users (HR: 0.51; 95% CI: 0.26, 1.00) (p-trend: 0.06). The association between fish oil supplement use and CRC risk remains relatively unexplored in the literature, with the association only previously assessed in the VITAL cohort (14). In an analysis following persons for CRC through 2006, Satia *et al* reported that any use of fish oil supplements in the 10-year period prior to baseline was associated with 35% reduced risk of CRC (95% CI:1%, 58%) (14). Here, we have 2 additional years of follow-up, providing more statistical power to assess a dose-response relationship and subgroup and subsite specific differences, though inclusion of additional follow-up likely increased measurement error, as cohort members may have changed supplement use over time. A few studies have reported on the association between total (diet+supplement) long-chain omega-3 intake and CRC either by questionnaire of diet and supplement use or by use of blood biomarkers. Results from the questionnaire-based study suggest a significant inverse association between total (dietary+supplement) EPA intake and CRC (24), though results from two small nested case-control studies using blood

biomarkers are less strong, with one reporting a non-significant inverse association (25) and another reporting a non-significant inverse association among men only (26).

In our study, the association between fish oil supplement use and CRC risk varied by gender (p-interaction:0.02) and by anatomic subsite (p-difference:0.05), with significant association observed among men (p-trend:0.02), but not among women. Results from prior epidemiologic studies have been inconsistent, with two reporting an inverse association among men only (26, 27), one an inverse association for women only (28), and another reporting no difference by gender (29). We also observed the association between fish oil supplement use and cancer risk to vary over anatomic subsite, with increasing fish oil use supplement use associated with reduced risk of colon cancer (p-trend: 0.03), but not associated with risk of rectal cancer. Given that aspirin is more strongly associated with colon cancer than rectal cancer(30), observation of stronger association with colon cancer might be expected as both aspirin and fish oil supplements are thought to reduce inflammation through the cyclooxygenase pathway (30). Our finding is supported by a previous cohort study by Sazuki *et al* in which marine omega-3 PUFA intake was significantly associated with reduced risk of proximal colon cancer among women, but not rectal cancer, though significance of subsite-specific differences was not presented and no difference was apparent among men (28). However, the literature is not consistent on this issue, and these results stand in contrast to two meta-analyses of fish consumption and CRC risk, with one reporting a stronger effect estimate for cancers of the rectum than for the colon (10), and another reporting comparable associations across subsite (11).

Lastly, we observed a significant interaction between overall genetic risk based on previously identified CRC susceptibility loci and i) dark fish consumption (p-interaction:0.009), and ii) total EPA+DHA intake (p-interaction:0.02). In both of these analyses, inverse associations were limited to the low/moderate genetic risk groups while positive associations were observed in the high genetic risk group (Table 4).

Our observation of a positive association between long-chain omega-3 intake and CRC risk among persons with high genetic load is supported by a recent study conducted among persons with Lynch Syndrome (31), a form of hereditary CRC caused by mutations in DNA mismatch repair genes. In this prospective cohort, study authors observed that fish oil supplement users had a marginally significant 1.74-fold higher risk of colorectal tumor than non-users (95%CI: 0.96-3.16) (32). While the association between long-chain omega-3 intake and CRC has not been previously studied in terms of effect modification by overall genetic risk, it is notable that prior studies have also shown the association between omega-3 intake and cancer risk to be modified by genetic variation. For example, a recent case-control study suggested that genetic variation at a tagging SNP in the *PARP* gene may modify the association between marine omega-3 intake and rectal cancer, with inverse association observed between marine omega-3 intake and rectal cancer among persons of the wild-type, and positive association observed among those with variation at this SNP (33). Additional observational research suggests that genetic variation in inflammation-related genes may modify the associations between long-chain omega-3 intake and risk of cancers of the colon and rectum (34) and colon polyp formation (35). Our results, in combination with these prior studies, suggest that the association between long-chain omega-3 intake and CRC risk may vary by underlying genetic risk, a point which may merit further attention when considering the potential role of long-chain omega-3 PUFAs in cancer prevention.

Research indicates that long-chain omega-3 PUFAs have several biologic effects which may be relevant to CRC prevention. Animal models have demonstrated that omega-3 PUFA-rich diets reduce the release of inflammatory biomarker, PGE_2 (36), and despite initially inconclusive small trials (37, 38), two recent RCTs have shown omega-3 PUFA supplementation reduces inflammation in humans (4, 5). Epidemiologic studies corroborate this growing body of evidence, with a recent study observed that regular fish oil supplement users have significantly lower levels of inflammation measured by high-sensitivity C-reactive protein (hsCRP) than non-users (39). Omega-3 PUFAs are thought to reduce inflammation by

competitively inhibiting pro-inflammatory omega-6 PUFAs via competition for cyclooxygenase enzyme activity and by displacement of omega-6 PUFA stores from cell membranes (40-42). Furthermore, *in vitro* studies demonstrate that omega-3 PUFAs inhibit the activity of nuclear factor kappa B (NFkB), a transcription factor central to the inflammatory cascade and which has been implicated in the etiology of several cancers (42, 43).

Beyond posited anti-inflammatory effects, *in vitro* and animal studies suggest that these long-chain omega-3 PUFAs may also have several additional anti-neoplastic effects, including inhibition of tumor growth or increased apoptosis (6, 7, 44) and suppression of angiogenesis (9). Furthermore, animal models have demonstrated that omega-3 PUFAs reduce the incidence of azoxymethane-induced colon tumors in rats by increasing cell differentiation and apoptosis (44). A recent small randomized trial in humans suggests that these mechanisms may extend beyond the animal model: after 3 months of treatment, persons supplemented with EPA had decreased mucosal proliferation and increased apoptosis at colonoscopy as compared to those in the control group (8).

A limitation of this study is that we were unable to assess changes in exposure over follow-up. Given that fish oil supplement use has increased over recent years (45), we might expect more non-users to become users over time. If a genuine association exists, one would expect the lack of additional exposure assessment to attenuate the association towards the null. Another limitation is the narrow range of exposure for dietary variables, limiting our ability to create substantial exposure contrast and detect significant association, potentially explaining why the association between fish oil supplement dose and CRC risk is stronger than other observed associations. Furthermore, those with symptoms of CRC at baseline may have changed their diet, and in order to address this concern of reverse causality, we conducted a sensitivity analysis excluding the first two years of follow-up. The associations strengthened upon exclusion of early follow-up, with the association between fish oil supplement use

and CRC becoming statistically significant. Given that a substantial change was observed for fish oil supplement use, a behavior unlikely to be affected by early symptoms of CRC, it seems more likely that this difference is related to the etiologically relevant time frame. By including the first two years of follow-up, we may have diluted results by including cases unlikely to have been impacted by exposure at baseline. However, given the *a priori* decision to treat this as a sensitivity analysis, emphasis should be placed on the full cohort.

In addition to the above-listed limitations, our study has several strengths. Notably, we were able to assess the associations between CRC and fish oil supplement use and total (dietary+supplemental) intake of long-chain omega-3 PUFAs. Furthermore, the FFQ used to assess dietary intake was enriched to better ascertain fatty acid intake, while many previous studies have not considered non-fish sources of EPA and DHA and many have studied total fish intake with no distinction made between fish of high and low EPA/DHA content (23). Lastly, the use of a cohort specifically designed to study the association between supplement use and cancer provided detailed information on exposures and covariates of interest, including omega-6 intake and reasons for why persons may increase omega-3 intake. Apart from results of fish oil supplements, these methodologic advantages did not lead to stronger results than have been observed in similar studies.

In our study, we were able to examine both dietary and supplementary sources of omega-3 PUFAs in a large prospective study. Furthermore, we were able to examine these associations across anatomic subsite and across risk subgroups. These subgroup and subsite specific differences merit further attention, as it is possible that future preventive efforts may focus on specific subgroups. Given the relative popularity of fish oil supplements and the high doses of long-chain omega-3 PUFAs in these supplements, future research is needed to better understand the association between fish oil supplement use and CRC risk.

Table 1. Participant Characteristics and Age and Sex-Adjusted Risk Ratios of Colorectal Cancer Risk, the VITAL Study (*n*=68,109)

	Cohort n=68,109 *n* (%)	Cases n=488 *n (%)*	Age and Sex Adjusted Risk Ratios[a]	95% Confidence Interval
Demographic				
Age at Baseline (years)				
50- <55	16,383 (24.1)	49 (10.0)	1.00	Ref
55- <60	15,805 (23.2)	63 (12.9)	1.36	0.93, 1.97
60- <65	12,516 (18.4)	82 (16.8)	2.24	1.57, 3.19
65- <70	11,020 (16.2)	131 (26.8)	4.08	2.94, 5.68
70+	12,385 (18.2)	163 (33.4)	4.64	3.37, 6.38
Sex				
Female	34,745 (51.0)	230 (47.1)	1.00	Ref
Male	33,364 (49.0)	258 (52.9)	1.17	0.98, 1.40
Race				
White	63,409 (93.8)	447 (92.4)	1.00	Ref
Hispanic	551 (0.82)	2 (0.41)	0.64	0.16, 2.57
Black	749 (1.11)	13 (2.69)	2.57	1.48, 4.47
American Indian/ Alaska Native	987 (1.46)	12 (2.48)	1.93	1.09, 3.43
Asian or Pacific Islander	1,508 (2.23)	7 (1.45)	0.69	0.33, 1.46
Other	398 (0.59)	3 (0.62)	1.17	0.38, 3.64
Education				
High School Graduate/GED or Less	12,836 (18.9)	145 (30.0)	1.00	Ref
Some College/ Technical School	25,745 (38.1)	184 (38.0)	0.75	0.60, 0.93
College Grad or Above	29,050 (43.0)	155 (32.0)	0.57	0.45, 0.72
Lifestyle/Diet				
Body Mass Index (m/kg^2)				
Normal Weight (<25)	22,769 (34.1)	141 (29.6)	1.00	Ref
Overweight (≥25-<30)	27,750 (41.3)	189 (39.6)	1.09	0.88, 1.36
Obese (≥30-<35)	11,053 (16.6)	96 (20.1)	1.51	1.17, 1.97
Severely Obese (≥35)	5,358 (8.03)	51 (10.7)	1.92	1.39, 2.65
Physical Activity (MET-hrs per week mod/vig activity)[b]				
None	34,365 (51.1)	294 (61.5)	1.00	Ref
Tertile 1	11,120 (16.5)	64 (13.4)	0.73	0.56, 0.96
Tertile 2	10,928 (16.3)	58 (12.1)	0.67	0.50, 0.89
Tertile 3	10,834 (16.1)	62 (13.0)	0.72	0.54, 0.94
Smoking Status				
Never Smoker	32,256 (47.8)	193 (40.0)	1.00	Ref
Former Smoker (Quit 10+ Yrs Prior)	25,160 (37.3)	196 (40.7)	1.13	0.92, 1.39
Former Smoker (Quit <10 Yrs Prior)	4,516 (6.70)	42 (8.71)	1.67	1.19, 2.33
Current Smoker	5,543 (8.21)	51 (10.6)	1.73	1.27, 2.36
Alcohol Intake (drinks)				
None- <1/month	24,977 (36.7)	181 (37.1)	1.00	Ref
1/month -≤4/week	20,213 (29.7)	128 (26.2)	0.93	0.74, 1.16
>4/week -<2/day	14,988 (22.0)	91 (18.7)	0.85	0.66, 1.10
2+/day	7,931 (11.6)	88 (18.0)	1.48	1.14, 1.93
Multivitamin Use				
Never	23,448 (34.4)	177 (36.3)	1.00	Ref
Past Only	5,128 (7.53)	34 (6.97)	0.96	0.66, 1.38
Current	39,525 (58.4)	277 (56.8)	0.89	0.74, 1.08
Calcium Intake (Diet + Supplemental) (mg/day)				
Quartile 1: <724.7	16,923 (25.0)	149 (30.7)	1.00	Ref
Quartile 2: 724.7 -<1036.4	16,923 (25.0)	117 (24.1)	0.77	0.61, 0.99
Quartile 3: 1036.4- <1462.0	16,923 (25.0)	108 (22.2)	0.71	0.55, 0.91
Quartile 4: 1462.0+	16,923 (25.0)	112 (23.1)	0.70	0.55, 0.90

Dietary Fiber Intake (g/day)				
Quartile 1: <12.4	17,028 (25.0)	129 (26.4)	1.00	Ref
Quartile 2: 12.4-<17.4	17,027 (25.0)	140 (28.7)	1.01	0.79, 1.29
Quartile 3: 17.4-<23.7	17,027 (25.0)	116 (23.8)	0.80	0.62, 1.04
Quartile 4: 23.7+	17,027 (25.0)	103 (21.1)	0.68	0.52, 0.90
Fruit/Vegetable Intake (servings/day)				
Quartile 1: <2.0	17,028 (25.0)	138 (28.3)	1.00	Ref
Quartile 2: 2.0-<3.2	17,027 (25.0)	137 (28.1)	0.95	0.75, 1.20
Quartile 3: 3.2-<4.8	17,027 (25.0)	107 (21.9)	0.72	0.56, 0.92
Quartile 4: 4.8+	17,027 (25.0)	106 (21.7)	0.73	0.57, 0.95
Red/ Processed Meat Intake (oz/week)				
Quartile 1: <9.11	17,028 (25.0)	103 (21.1)	1.00	Ref
Quartile 2: 9.11- <17.1	17,027 (25.0)	133 (27.3)	1.29	1.00, 1.67
Quartile 3: 17.1- <28.3	17,027 (25.0)	111 (22.8)	1.09	0.83, 1.43
Quartile 4: 28.3+	17,027 (25.0)	141 (28.9)	1.42	1.09, 1.87
Omega-6 PUFAs (Linoleic + Arachidonic) (g/day)				
Quartile 1: <8.29	17,028 (25.0)	132 (27.1)	1.00	Ref
Quartile 2 8.29- <12.3	17,027 (25.0)	114 (23.4)	0.85	0.66, 1.09
Quartile 3 12.3- <18.0	17,027 (25.0)	110 (22.5)	0.79	0.61, 1.03
Quartile 4: 18.0+	17,027 (25.0)	132 (27.1)	0.94	0.72, 1.23
Medication				
Aspirin Use[c]				
Non-user	36,345 (54.8)	284 (59.9)	1.00	Ref
Low	15,636 (23.6)	106 (22.4)	0.77	0.62, 0.96
High	14,331 (21.6)	84 (17.7)	0.55	0.43, 0.70
Non-aspirin NSAID Use[c]				
Non-user	46,503 (69.6)	372 (78.2)	1.00	Ref
Low	15,646 (23.4)	78 (16.4)	0.69	0.54, 0.89
High	4,650 (7.00)	26 (5.46)	0.76	0.51, 1.13
Hormone Replacement Therapy				
Never	11,594 (36.3)	88 (40.7)	1.00	Ref
Former	4,873 (15.3)	41 (19.0)	0.86	0.59, 1.25
Current	15,469 (48.4)	87 (40.3)	0.72	0.53, 0.97
Other				
Family History of Colorectal Cancer				
None	59,565 (88.6)	407 (85.3)	1.00	Ref
1+ 1st Degree Relative	7,693 (11.4)	70 (14.7)	1.24	0.96, 1.60
History of Sigmoidoscopy/Colonoscopy (last 10 yrs)				
No	29,581 (43.8)	257 (53.3)	1.00	Ref
Yes	38,025 (56.3)	225 (46.7)	0.52	0.44, 0.63
History of Polyp Excision				
No	59,717 (87.7)	423 (86.7)	1.00	Ref
Yes	8,392 (12.3)	65 (13.3)	0.88	0.67, 1.14
Genetic Risk Score (number risk alleles)[d]				
Lowest Risk (<14.5)	170 (33.3)	74 (29.6)	1.00	Ref
Mid Risk (14.5- <16.9)	170 (33.3)	84 (33.6)	1.28	0.83. 1.98
High Risk (16.9+)	170 (33.3)	92 (36.8)	1.52	0.99, 2.33

Abbreviations: 95% CI (95% confidence interval)

[a] Hazard ratios presented for all risk factors except genetic risk score, for which odds ratios are presented

[b] Tertiles of physical activity among those engaging in moderate/vigorous leisure time physical activity determined within gender; women (T1: <2.81; T2: 2.81-<9.48; T3: 9.48+); men (T1: <-4.38; T2: 4.38- <13.3; T3: 13.3+)

[c] Use of aspirin (including both low-dose and regular aspirin) and non-aspirin NSAID defined by use over 10 years prior to baseline, with low use defined as use <4 days per week or <4 years, and high use defined as 4+ days per week for 4+ years

[d] Genetic risk score determined by risk score generated from 16 single nucleotide polymorphisms associated with colorectal cancer risk (250 cases, 260 controls); in addition to adjusting for age and sex, genetic risk scores are additionally adjusted for population substructure

Table 2. Hazard Ratios (HR) of Colorectal Cancer Associated with Fish Oil Supplement Use, Dark Fish Consumption, and Long-Chain Omega-3 Polyunsaturated Fatty Acid Intake

	Cohort	Cases	Age and Sex Adjusted		Multivariate Adjusted[a]	
	n=68,109 N (%)	*n*=488 N (%)	HR	95% CI	HR	95% CI
Fish Oil Supplement Use[b]						
Average 10-yr use						
No Use	61,936 (90.1)	456 (93.4)	1.00	Ref	1.00	Ref
Low Use (<4 days/week or <3 yrs)	3,806 (5.59)	21 (4.30)	0.78	0.51, 1.22	0.93	0.59, 1.49
High Use (≥4 days/week and ≥3 yrs)	2,907 (4.27)	11 (2.25)	0.48	0.26, 0.87	0.51	0.26, 1.00
			P-trend: 0.009		*P-trend: 0.06*	
Dietary Fish Consumption[b]						
Dark Fish (Salmon +Tuna) (serv/wk)						
Quartile 1: None	24,021 (35.3)	202 (41.4)	1.00	Ref	1.00	Ref
Quartile 2: >0 - <0.26	15,978 (23.5)	107 (21.9)	0.79	0.62, 0.99	0.88	0.68, 1.14
Quartile 3: 0.26-<0.80	16,178 (23.8)	118 (24.2)	0.85	0.68, 1.07	1.07	0.83, 1.38
Quartile 4: 0.80+	11,932 (17.5)	61 (12.5)	0.62	0.46, 0.82	0.77	0.55, 1.07
			P-trend: 0.002		*P-trend: 0.40*	
Eicosapentaenoic Acid (EPA)[b]						
Dietary EPA (g/day)						
Quartile 1: <0.02	17,028 (25.0)	141 (28.9)	1.00	Ref	1.00	Ref
Quartile 2: 0.02-<0.05	17,027 (25.0)	118 (24.2)	0.82	0.64, 1.05	0.89	0.68, 1.17
Quartile 3: 0.05-<0.09	17,027 (25.0)	111 (22.8)	0.77	0.60, 0.99	0.93	0.70, 1.23
Quartile 4: 0.09+	17,027 (25.0)	118 (24.2)	0.79	0.62, 1.02	0.99	0.74, 1.33
			P-trend: 0.05		*P-trend: 1.00*	
Total EPA[d] (g/day)						
Quartile 1: <0.03	17,029 (25.0)	146 (29.9)	1.00	Ref	1.00	Ref
Quartile 2: 0.03-<0.06	17,026 (25.0)	116 (23.8)	0.78	0.61, 1.00	0.86	0.66, 1.13
Quartile 3: 0.06-<0.11	17,027 (25.0)	117 (24.0)	0.78	0.61, 1.00	0.96	0.72, 1.26
Quartile 4: 0.11+	17,027 (25.0)	109 (22.3)	0.71	0.55, 0.91	0.91	0.67, 1.22
			P-trend: 0.01		*P-trend: 0.67*	
Docosahexaenoic Acid (DHA)[b]						
Dietary DHA (g/day)						
Quartile 1: <0.05	17,028 (25.0)	146 (29.9)	1.00	Ref	1.00	Ref
Quartile 2: 0.05-<0.11	17,027 (25.0)	122 (25.0)	0.82	0.64, 1.04	0.90	0.69, 1.17
Quartile 3: 0.11-<0.19	17,027 (25.0)	112 (23.0)	0.75	0.59, 0.96	0.94	0.71, 1.23
Quartile 4: 0.19+	17,027 (25.0)	108 (22.1)	0.71	0.55, 0.91	0.89	0.66, 1.21
			P-trend: 0.005		*P-trend: 0.56*	
Total DHA[d](g/day)						
Quartile 1: <0.05	17,028 (25.0)	145 (29.7)	1.00	Ref	1.00	Ref
Quartile 2: 0.05-<0.11	17,027 (25.0)	127 (26.0)	0.86	0.68, 1.09	0.96	0.74, 1.25
Quartile 3: 0.11-<0.21	17,027 (25.0)	116 (23.8)	0.78	0.61, 1.00	0.98	0.74, 1.29
Quartile 4: 0.21+	17,027 (25.0)	100 (20.5)	0.66	0.51, 0.85	0.86	0.63, 1.16
			P-trend: 0.001		*P-trend: 0.38*	
EPA + DHA[b]						
Dietary EPA + DHA (g/day)						
Quartile 1: <0.08	17,123 (25.1)	145 (29.7)	1.00	Ref	1.00	Ref
Quartile 2: 0.08- <0.16	17,103 (25.1)	124 (25.4)	0.84	0.66, 1.06	0.91	0.70, 1.19
Quartile 3: 0.16- <0.29	16,870 (24.8)	110 (22.5)	0.75	0.58, 0.96	0.92	0.69, 1.22
Quartile 4: 0.29+	17,013 (25.0)	109 (22.3)	0.72	0.56, 0.93	0.92	0.68, 1.24
			P-trend: 0.007		*P-trend: 0.61*	
Total EPA +DHA[d] (g/day)						
Quartile 1: <0.08	17,134 (25.2)	142 (29.1)	1.00	Ref	1.00	Ref
Quartile 2: 0.08- <0.17	16,955 (24.9)	126 (25.8)	0.88	0.69, 1.12	1.00	0.77, 1.31
Quartile 3: 0.17- <0.32	17,037 (25.0)	120 (24.6)	0.83	0.65, 1.06	1.07	0.81, 1.41
Quartile 4: 0.32+	16,983 (24.9)	100 (20.5)	0.67	0.52, 0.87	0.88	0.65, 1.20
			P-trend: 0.003		*P-trend: 0.56*	
Total EPA/DHA to Omega-6 Ratio[b,c]						
Quartile 1: <0.007	17,007 (25.0)	141 (28.9)	1.00	Ref	1.00	Ref
Quartile 2: 0.007- <0.01	17,021 (25.0)	137 (28.1)	0.96	0.76, 1.21	1.06	0.82, 1.38
Quartile 3: 0.01- <0.03	17,047 (25.0)	113 (23.2)	0.79	0.62, 1.02	1.01	0.77, 1.33
Quartile 4: 0.03+	17,034 (25.0)	97 (19.9)	0.68	0.52, 0.88	0.85	0.63, 1.16
			P-trend: 0.001		*P-trend: 0.33*	

Abbreviations: DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); HR (hazard ratio); 95% CI (95% confidence interval)

[a] Multivariate analyses include 59,500 study participants, including 419 cases

[b] Multivariate model adjusted for age, sex, race/ethnicity, education, BMI, energy intake, MET-hours per week of moderate/vigorous activity, alcohol intake, smoking history, multivitamin use, calcium intake, dietary fiber intake, fruit and vegetable, red/processed meat, aspirin use, non-aspirin NSAID use, family history of colorectal cancer, history of sigmoidoscopy/ colonoscopy, history of polyps, hormone replacement therapy, cardiovascular disease, memory loss, use of cholesterol-lowering drugs, and omega-6 (linoleic +arachidonic) intake

[c] Ratio of (total EPA + DHA) to (lineolic acid + arachidonic acid)

[d] Includes both dietary intake and supplementary intake (estimated from fish oil of supplement use)

Table 3. Hazard Ratios (HR) of Colorectal Cancer Associated with Fish Oil Supplement Use, Dark Fish Consumption, and Total EPA+DHA, by Gender

	Male			Female			P-interaction
	Case/Cohort	HR	95% CI	Case/ Cohort	HR	95% CI	
10-yr Fish Oil Use[a]							
No Use	224/27,654	1.00	Ref	167/26,000	1.00	Ref	0.02
Low Use (<4 days/week or <3 years)	6/1,410	0.66	0.29,1.49	13/1,891	1.16	0.66,2.05	
High Use (≥4 days/week and ≥3 yrs)	2/1,271	0.22	0.06,0.90	7/1,274	0.85	0.39,1.80	
		P-trend: 0.02			*P-trend: 0.88*		
Dark Fish (Salmon +Tuna) (serv/wk)[a]							
Quartile 1: None	94/10,409	1.00	Ref	77/10,440	1.00	Ref	0.87
Quartile 2: >0 - <0.26	50/7,300	0.82	0.58,1.16	42/6,687	0.96	0.65,1.41	
Quartile 3: 0.26-<0.80	64/7,205	1.18	0.85,1.64	42/6,956	0.91	0.62,1.34	
Quartile 4: 0.80+	24/5,421	0.69	0.43,1.11	26/5,082	0.86	0.54,1.38	
		P-trend: 0.57			*P-trend: 0.49*		
Total EPA +DHA (g/day)[b]							
Quartile 1: <0.08	54/5,719	1.00	Ref	61/9,044	1.00	Ref	0.72
Quartile 2: 0.08- <0.17	50/6,588	0.84	0.57,1.25	58/8,131	1.14	0.79,1.65	
Quartile 3: 0.17- <0.32	67/8,187	1.01	0.70,1.47	43/6,824	1.08	0.72,1.64	
Quartile 4: 0.32+	61/9,841	0.89	0.59,1.32	25/5,166	0.82	0.50,1.34	
		P-trend: 0.78			*P-trend: 0.56*		

Abbreviations: DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); HR (hazard ratio); 95% CI (95% confidence interval)

[a] Multivariate model adjusted for age, sex, race/ethnicity, education, BMI, energy intake, MET-hours per week of moderate/vigorous activity, alcohol intake, smoking history, multivitamin use, calcium intake, dietary fiber intake, fruit and vegetable intake, red/processed meat intake, aspirin use, non-aspirin NSAID use, family history of colorectal cancer, history of sigmoidoscopy/colonoscopy, history of polyps, hormone replacement therapy, cardiovascular disease, memory loss, use of cholesterol-lowering drugs, and omega-6 (linoleic +arachidonic) intake

[b] Includes both dietary intake and supplementary intake (estimated from fish oil supplement use)

Table 4. Odds Ratios (OR) of Colorectal Cancer Associated with Dark Fish Consumption and Total EPA+DHA Intake, by Genetic Risk[a]

	Overall			Low Genetic Risk			Mid-Genetic Risk			High-Genetic Risk			
	Case/ Control	OR	95% CI	Case/ Control	OR	95% CI	Case/ Control	OR	95% CI	Case/ Control	OR	95% CI	P
Dark Fish (serv/wk)[b]													
Quartile 1: None	91/76	1.00	Ref	29/27	1.00	Ref	30/21	1.00	Ref	32/28	1.00	Ref	*0.009*
Quartile 2: >0 -<0.26	41/53	0.62	0.37,1.06	14/18	0.63	0.24,1.67	10/18	0.25	0.08,0.75	17/17	0.92	0.35,2.39	
Quartile 3:0.26<0.80	59/54	0.87	0.52,1.44	14/23	0.47	0.19,1.21	24/21	0.55	0.21,1.49	21/10	2.16	0.80,5.80	
Quartile 4: 0.80+	25/43	0.41	0.22,0.76	5/19	0.13	0.04,0.48	8/15	0.14	0.04,0.53	12/9	1.59	0.51,4.97	
		p-trend:0.02			*p-trend:0.002*			*p-trend:0.01*			*p-trend:0.17*		
Total EPA +DHA (g/day)[b,c]													
Quartile 1: <0.08	58/60	1.00	Ref	16/22	1.00	Ref	23/18	1.00	Ref	19/20	1.00	Ref	*0.02*
Quartile 2:0.08<0.17	60/54	1.15	0.68,1.97	20/18	1.40	0.51,3.87	17/20	0.69	0.26,1.81	23/16	1.82	0.66,5.00	
Quartile 3: 0.17<0.32	48/58	0.82	0.47,1.44	16/20	0.84	0.30,2.34	17/20	0.36	0.12,1.05	15/18	1.07	0.37,3.10	
Quartile 4: 0.32+	50/54	0.92	1.51,1.66	10/27	0.23	0.07,0.78	15/17	0.43	0.12,1.41	25/10	5.79	1.79,18.7	
		p-trend: 0.54			*p-trend:0.02*			*p-trend:0.08*			*p-trend:0.01*		

Abbreviations: DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); OR (odds ratio); 95% CI (95% confidence interval)

[a] Genetic risk defined by number of risk alleles at 16 SNPs at susceptibility loci shown to be associated with colorectal cancer risk (see Methods)

[b] Adjusted for age, sex, principal components (population substructure), energy intake, alcohol intake (see Methods)

[c] Includes both dietary intake and supplementary intake (estimated from fish oil supplement us

Table 5. Hazard Ratios (HR) of Colorectal Cancer Associated with Fish Oil Supplement Use, Dark Fish Consumption, and Total EPA+DHA Intake, by Subsite

	Colon Cancer (n=311)			Rectal Cancer (n=108)			P for difference
	Case/Cohort	HR	95% CI	Case/Cohort	HR	95% CI	
10-yr Fish Oil Use[a]							
No Use	293/53,654	1.00	Ref	98/53,654	1.00	Ref	0.05
Low Use (<4 days/week or <3 yrs)	13/3,301	0.84	0.48,1.47	6/3,301	1.22	0.53,2.82	
High Use (≥4 days/week and ≥3 yrs)	5/2,545	0.37	0.15,0.91	4/2,545	0.98	0.35,2.69	
		P trend: 0.03			*P trend: 0.87*		
Dark Fish (Salmon +Tuna): (servings/week)[a]							
Quartile 1: None	126/20,849	1.00	Ref	45/20,849	1.00	Ref	0.46
Quartile 2: >0 - <0.26	67/13,987	0.88	0.65,1.19	25/13,987	0.86	0.53,1.42	
Quartile 3: 0.26-<0.80	85/14,161	1.18	0.88,1.57	21/14,161	0.77	0.45,1.33	
Quartile 4: 0.80+	33/10,503	0.71	0.47,1.06	17/10,503	0.90	0.50,1.64	
		P trend: 0.53			*P trend: 0.53*		
Total EPA +DHA (g/day)[b]							
Quartile 1: <0.08	90/14,763	1.00	Ref	25/14,763	1.00	Ref	0.39
Quartile 2: 0.08- <0.17	81/14,719	0.98	0.72,1.33	27/14,719	1.10	0.63,1.91	
Quartile 3: 0.17- <0.32	83/15,011	1.06	0.77,1.46	27/15,011	1.11	0.63,1.97	
Quartile 4: 0.32+	57/15,007	0.78	0.54,1.12	29/15,007	1.22	0.67,2.21	
		P trend:0.30			*P trend: 0.53*		

Abbreviations: DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); HR (hazard ratio); 95% CI (95% confidence interval)

[a] Multivariate model adjusted for age, sex, race/ethnicity, education, BMI, energy intake, MET-hours per week of moderate/vigorous activity, alcohol intake, smoking history, multivitamin use, calcium intake, dietary fiber intake, fruit and vegetable intake, red/processed meat intake, aspirin use, non-aspirin NSAID use, family history of colorectal cancer, history of sigmoidoscopy/colonoscopy, history of polyps, hormone replacement therapy, cardiovascular disease, memory loss, use of cholesterol-lowering drugs, and omega-6 (linoleic +arachidonic) intake

[b] Includes both dietary intake and supplementary intake (estimated from fish oil supplement use)

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Chapter 3. Specialty supplement use and biologic measures of oxidative stress and DNA damage

ABSTRACT

Oxidative stress and resulting cellular damage have been associated with increased risk of several common chronic diseases including cancer and cardiovascular disease. Identifying ways of reducing oxidative stress and resulting damage may reduce risk of these diseases. In the VITamins And Lifestyle (VITAL) biomarker study of 220 persons living in the Seattle area, we examined the association between use of several specialty supplements [glucosamine, chondroitin, fish oil, methylsulfonylmethane (MSM), co-enzyme Q10 (CoQ10), ginseng, ginkgo, saw palmetto, and fiber] and measures of oxidative stress, DNA damage, and DNA repair capacity. Use of glucosamine, chondroitin, fish oil, MSM, CoQ10, ginseng, ginkgo, and saw palmetto was ascertained by a supplement inventory /interview, while fiber was ascertained by questionnaire. Oxidative stress was measured by urinary 8-isoprostane and PGF2α concentrations using an ELISA, while leukocyte DNA damage and repair capacity at 15 minutes and 60 minutes were measured using the comet assay. Multivariate-adjusted linear regression was used to model the association between specialty supplement use and measures of oxidative stress/DNA damage. Use of glucosamine (p-trend: 0.01), chondroitin (p-trend: 0.003), and fiber supplements (p: 0.01) were associated with reduced PGF2α concentrations, while CoQ10 supplementation was associated with reduced baseline DNA damage (p: 0.001). Results suggest that use of certain specialty supplements may be associated with reduced oxidative stress and DNA damage.

INTRODUCTION

In a state of oxidative stress, excess reactive species can act to damage various cellular components, including membrane lipids and DNA (1-3). Oxidative stress and resulting DNA damage have been suggested to play a role in several adverse health outcomes (4), including cardiovascular disease (5, 6) and certain cancers (3, 6-12), though prospective evidence of an association remains limited. It is therefore possible that reducing oxidative stress and preventing the ensuing cellular damage may offer a possible disease prevention strategy.

While non-vitamin, non-mineral specialty supplements are often used for disease prevention, the mechanisms by which these supplements might reduce risk of diseases are not well studied in humans. Specialty supplements, such as glucosamine (13, 14), chondroitin (15, 16), and co-enzyme Q10 (CoQ10) (17-19) have been suggested to reduce oxidative stress and/or DNA damage in laboratory studies. Despite suggestive *in vitro* and animal studies, the association between glucosamine and chondroitin and oxidative stress/DNA damage has not been studied in humans, and evidence of an effect of CoQ10 on oxidative stress/DNA damage is conflicting (20-28). Though little is known about the effects of specialty supplements on oxidative stress, we might hypothesize that supplements with suggested anti-inflammatory properties may be associated with reduced oxidative stress and DNA damage given the close association between these two biologic processes.

Given the limited literature on the association between specialty supplement use and oxidative stress, we have examined the associations between use of specialty supplements posited to have anti-oxidant or anti-inflammatory properties [glucosamine (29-31), chondroitin (29, 31, 32), fish oil (31, 33, 34), CoQ10 (35), methylsulfonylmethane (MSM) (36), garlic (37-39), ginseng (40, 41), ginkgo (42), saw palmetto (43), and fiber (44)] and oxidative stress/ DNA damage in the VITamins And Lifestyle (VITAL)

biomarker study. Oxidative stress has been measured by urinary 8-isoprostane and PGF2α and leukocyte DNA damage and DNA repair capacity have been measured by the comet assay.

METHODS

Study Population

Study participants were drawn from the VITAL biomarker study, a subset of 220 VITAL cohort participants who completed the 24-page mailed VITAL baseline questionnaire between October 2000 and February 2001. VITAL is a prospective study of 77,719 western Washington residents aged of 50-76 followed for cancer incidence and mortality (45). Biomarker study participants were randomly selected from the VITAL cohort so as to obtain an equal sex distribution, while oversampling high users of vitamin C, vitamin E, and calcium supplements. Persons living outside the Seattle metropolitan area were excluded from the biomarker study, as were persons with insulin-dependent diabetes or any conditions preventing the collection of fasting blood. Two hundred and ninety persons were contacted for participation in the biomarker study, 220 (76%) of whom agreed to participate and completed the study protocol. Biomarker study participants completed a second mailed questionnaire (approximately 3 -5 months after completing the baseline VITAL questionnaire), and participated in an in-home interview. Blood and urine was collected at the home interview, as was more detailed information on several factors, including supplement use. All study procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board and all study participants provided written informed consent.

Exposure--Use of specialty supplements

Participants' use of supplements was ascertained at the time of the home visit, at which time a supplement inventory/interview was conducted. Participants were asked to report on the frequency (times/week) of supplements used, the number of pills taken per occasion of use, and the date at which each supplement was last used. Persons reporting use of a given supplement within the two weeks prior to interview were classified as current users, while those reporting no use or last use more than two

weeks prior to interview/ specimen collection were classified as non-users. It should be noted that most persons classified as "current users" reported use within the day prior; for example, 85% of persons classified as "current glucosamine users" reported most recent use within the day prior. This supplement inventory/ home interview provided the source of information on the following supplements: glucosamine, chondroitin, fish oil, CoQ10, MSM, garlic, ginseng, ginkgo, and saw palmetto (men only).

Unlike the other supplements included in this study, use of fiber supplements was not ascertained at the time of home interview/ supplement inventory, but was instead ascertained as part of the mailed biomarker study questionnaire. Participants were asked to report whether fiber-containing supplements were used at least once a week over the prior month. Those responding "yes" were classified as current users, while those reporting "no" were classified as non-users.

For supplements used by more than 30 persons (glucosamine and chondroitin), we calculated the average number of pills/week of use [non-users, low users (<14 pills per week), or high users (14+ pills per week)]. Persons missing information on the number of pills taken per occasion of use (n=7 for glucosamine, n=5 for chondroitin) were assumed to use 1 pill/time so as to allow for calculation of pills/week. This value was chosen for imputation, as the majority study participants reporting use of glucosamine and chondroitin (75% and 80%, respectively) reported 1 pill per occasion of use. For supplements used by less than 30 participants, we classified persons as either current users or non-users in order to preserve power.

Potential confounders

We decided *a priori* to adjust all analyses for age (continuous), sex, and pack-years smoked (ordinal: non-smokers, smokers with pack-years below median, smokers with pack years above the median). Additional potential confounding factors were evaluated by assessing the association between each

potential confounder (listed below) and each outcome (8-isoprostane, PGF2α, baseline DNA damage, and DNA repair at 15 minutes and 60 minutes) in a model adjusted for age, sex and pack-years of smoking. Variables associated with a given outcome at the alpha=0.10 level in this minimally-adjusted model were included as covariates in the final model of the association between specialty supplements and that outcome. This threshold of significance was selected so as to err on the side of inclusion, rather than exclusion, of potential confounders. This process of selecting covariates was performed for each of the 5 outcomes separately.

We evaluated the following variables as potential confounders: demographic factors [race/ethnicity (non-white/white), education (ordinal: HS grad/GED or less, some college/tech, college grad, advanced degree)] , lifestyle/anthropometric factors [body mass index (ordinal: <25 kg/m^2, 25-<30 kg/m^2, 30+ kg/m^2), alcohol consumption (indicator: tertiles), moderate/vigorous physical activity in the last month (none/any)], and medical factors [current regular aspirin (none, <4 days/week, 4+ days/week), current regular baby aspirin (none, <4 days/week, 4+ days/week), current regular non-aspirin NSAIDs (none, <4 days/week, 4+ days/week), current HRT (no/yes), cholesterol-lowering drugs (no/yes: use in the last two weeks), history of cardiovascular disease (no/yes; includes: coronary bypass surgery, angioplasty, angina, myocardial infarction, stroke), history of cancer (no/yes; excludes non-melanoma skin cancers), history of diabetes (no/yes), and history of sunburns (no/yes: 3+ severe sunburns between the ages of 10-20)]. We also evaluated use of multivitamins (no/yes) and intake of the following vitamins and minerals from supplements and from diet and supplements combined: beta-carotene, vitamin C, alpha-tocopherol, iron, selenium, and zinc. Additional dietary factors considered include energy intake (continuous), fiber intake (continuous), saturated fat intake (continuous), and dietary gamma tocopherol intake (continuous). In evaluating the association of dietary and diet plus supplemental intake with the biomarker outcomes, energy intake was included in the model. Since baseline DNA damage may plausibly reflect long-term exposures from accumulating insults, we also tested a select subset of

historical variables representing long-term exposure for this outcome, including BMI at age 45, 10-year physical activity, as well as 10-year supplemental intake of beta-carotene, vitamin C, and alpha-tocopherol.

For smoking and BMI variables, we averaged estimates from the two questionnaires (baseline VITAL questionnaire and biomarker questionnaire). Physical activity was ascertained in two ways, with one variable corresponding to current physical activity (part of the biomarker questionnaire) and the other corresponding to physical activity over the 10 years prior to baseline (part of the main VITAL questionnaire). On the biomarker questionnaire, participants were asked whether they engaged in any moderate or strenuous activity (activities with metabolic equivalent of task ≥4) over the prior month Those indicating that they had engaged in such activity at least once per week over the last month were classified as engaging as any moderate/vigorous physical activity, as were those indicating that they walked at a fast pace for exercise at least once per week over the last month. As noted above, we also assessed the association between 10-year physical activity and baseline DNA damage. Moderate/vigorous activity over the 10-year period prior to baseline was ascertained in the same way (on the baseline questionnaire), but instead participants were asked to consider the 10 year period prior to baseline.

Intake of supplemental vitamins and minerals was ascertained at the time of home interview. Respondents provided information on the frequency of each supplement used, as well as the number of pills taken per occasion of use. The interviewer also transcribed the dose of each vitamin/mineral contained in each pill from supplement bottle labels. From this information, we computed supplemental intake per day of each nutrient from multivitamins and individual supplements. Intake was categorized into three groups: no use, low use, and high use. The threshold between low use and high use was set

specifically for each supplement so as to ensure that high dose intake could not be obtained by using only common-formulation multivitamins (e.g., Centrum). These specific thresholds are listed in Table 1.

Dietary factors were ascertained by a food frequency questionnaire (FFQ) as part of the baseline VITAL questionnaire and of the biomarker study questionnaire. The nutrient intakes from the two questionnaires were averaged to reduce measurement error. The FFQ used in VITAL ascertained information on the frequency and serving size of 120 food and beverage items, and is based on the FFQ used in the Women's Health Initiative (46). Values for all other covariates were ascertained using the biomarker study questionnaire. In the event that we were missing information on a given covariate in the biomarker questionnaire, information was "borrowed" from the baseline VITAL questionnaire provided that the variables were ascertained in the same manner across questionnaires.

We also adjusted for conditions which may have increased use of supplements, regardless of their association with the outcomes. All multivariate-adjusted models pertaining to glucosamine, chondroitin, and MSM were further adjusted for self-reported history of arthritis or chronic pain. For fish oil, we further adjusted for history of cardiovascular disease (includes any of the following conditions: coronary bypass surgery, angioplasty, angina, myocardial infarction, or stroke) and memory loss (memory better/same as age 25, memory worse than age 25). For ginkgo and ginseng, we also adjusted for memory loss (memory better/same as age 25, memory worse than age 25). We adjusted for benign prostatic hyperplasia in analyses of saw palmetto, and for constipation in analyses of fiber supplements (as defined by constipation 1+ times/month).

Biomarker Outcomes

Oxidative stress was measured by 8-isoprostane and PGF2α. 8-isoprostane is a type of F2-isoprostane commonly used to measure lipid peroxidation, and is formed when reactive species initiate the peroxidation of arachidonic acid stored in the cell membrane in a cyclooxygenase (COX)-independent

process (2, 3) (47). Recent work has demonstrated that urinary PGF2α is formed by a similar COX-independent isoprostane pathway, and can also be used as a measure of oxidative stress (48). 8-isoprostane and PGF2α were assayed by enzyme-linked immunosorbent assay (ELISA; Cayman Chemical kits #516351 and #516011, respectively) using unacidified urine collected at the time of home interview. Urine was processed within approximately 2 hours of collection at the Fred Hutchinson Cancer Research Center Specimen Processing Laboratory and was frozen at -80°C thereafter. Both 8-isoprostane and PGF2α were reported in terms of pg/mL of urine, and the assays were reported to have a lower level of detection of 2.7 pg/mL for 8-isoprostane and 9 pg/mL for PGF2α. For PGF2α, one value fell below the level of quantification and was replaced with one half of the lowest detected value. ELISAs were conducted at the German National Cancer Institute (Heidelberg, Germany) and plates were read using the μQuant spectrophotometer from Bio-Tek Instruments (410 nm). Both the 8-isoprostane and PGF2α assays were corrected for urinary creatinine (mg/mL), and values are therefore expressed as pg/mg creatinine.

We have also measured DNA damage, as reactive species resulting from oxidative stress can also cause DNA breaks to occur. Such damage can persist either unrepaired or may be repaired via DNA repair mechanisms (6), though these repair mechanisms, too, may also be affected by oxidative stress (49). Furthermore, since it is DNA damage in the presence of diminished DNA repair capacity which has been implicated in the development of disease (50, 51), we have also measured DNA repair capacity. Baseline DNA damage and DNA repair capacity were assessed using leukocytes isolated from semi-fasting (6+ hours) blood samples collected at the time of home interview. Blood was processed within approximately 2 hours of collection at the Fred Hutchinson Cancer Research Center Specimen Processing Laboratory. A variation of the comet assay was used to quantify DNA damage (single strand and double strand breaks) (52-54); this assay was conducted at the German Cancer Research Center using the Metafer 4 system. In this single cell electrophoresis assay, damaged DNA fragments migrate

toward the anode (55). The migration of damaged DNA forms a tail, and the formation of a head and tail gives this assay the appearance of a comet, where the amount of migrated DNA represents the amount of DNA damage. In this study, baseline DNA damage was expressed as the olive tail moment, a parameter which is calculated by first subtracting the head mean from the tail mean, after which this difference is then multiplied by the percent of DNA in the tail/100 (55).

In order to measure DNA repair capacity, cells were exposed to damage (9 seconds of 1.23 gray of gamma radiation at 4°C). Fifteen minutes after damage had been induced (and again at 60 minutes), the percentage of damage repaired by that time point was calculated. The 15-minute repair capacity was calculated as follows: [1-(olive tail moment at 15 minutes/ olive tail moment immediately after irradiation)], and the 60-minute repair capacity was measured by [1-(olive tail moment at 60 minutes/ olive tail moment immediately after irradiation)] (55). These values represent the percentage of induced DNA damage repaired at each time point.

Of the 220 persons included in the biomarker study, 14 were excluded from analyses of 8-isoprostane and PGF2α due to lack of available urine to measure either the marker of interest or creatinine, leaving 206 persons for analyses corresponding to these measures. Thirty-five persons with a self-reported history of cancer were excluded from analyses of DNA damage and DNA repair capacity. An additional 63 persons were excluded from these DNA damage and DNA repair analyses because leukocytes were not viable, less than 60 cells were able to be scored, or if 50% or more of the cells were determined to be ghost cells (defined as the lack of cell viability 24 hours after being thawed). After making these exclusions, 122 persons remained eligible for analyses of baseline DNA damage and repair. An additional 23 persons were excluded from DNA repair capacity analyses due to having either i) implausibly higher baseline damage than induced damage, or ii) a higher level of residual damage than induced damage. After this final exclusion, 99 persons remained for analyses of DNA repair capacity.

Statistical analysis

Unadjusted Pearson correlation coefficients were used to assess the correlations between biomarkers (Table 1). Linear regression was used to evaluate the association of specialty supplement use and covariates with measures of oxidative stress and DNA damage. All specialty supplement exposures were modeled using either binary or indicator variables. The p-trend for variables with 3 or more exposure levels (glucosamine and chondroitin) was based on a model with the variable of interest modeled as an ordinal variable. Analyses presented in Table 2 are adjusted for age (continuous), sex, and pack-years smoked (none, below median, above median). Our final models as presented in Table 3 include covariates which were associated with a given outcome at the alpha=0.10 level in our minimally-adjusted model and are listed in the footnotes to Table 3.

Since distributions of 8-isoprostane, PGF2α, and baseline DNA damage were right-skewed, we log-transformed these variables to normalize their distributions. For these three log-transformed outcomes, we exponentiated our results so as to present the average geometric means per exposure category (Tables 2-3). The distributions of DNA repair capacity at 15 and 60 minutes were close to normally distributed, and we did not transform these variables and instead present means. In order to estimate the mean/geometric mean of each outcome per exposure level, covariates were fixed at their means. We have presented our results as means/geometric means (rather than betas/exponentiated betas) to keep the presentation and interpretation of results similar across outcomes.

While we included potential confounders with $p<0.10$, we used a more stringent alpha level to determine statistical significance of associations under study. We chose to compare results for the association between supplement use and markers of oxidative stress/DNA damage to a more conservative alpha level (alpha=0.01) in order to reduce the likelihood of false positive results. All analyses were conducted using Stata (version 12, College Station, TX).

RESULTS

In our study, the two measures of oxidative stress, PGF2a and 8-isoprostane, were modestly correlated (r=0.22), and neither of these measures were correlated with measures of DNA damage nor were they correlated with DNA repair capacity (Table 1). Baseline DNA damage was modestly negatively correlated with DNA repair capacity at 15 minutes and 60 minutes (r=-0.30 and -0.27, respectively) and DNA repair capacity at 15 minutes and 60 minutes were positively correlated (r=0.35).

In minimally-adjusted models, female gender (p: 0.02), low education (p-trend: 0.001), smoking (p-trend: 0.002), and increasing BMI (p-trend: 0.09) were associated with increased 8-isoprostane concentrations (Table 2). Use of beta-carotene and vitamin E supplements were associated with reduced 8-isoprostane concentrations (p-trend: 0.06, 0.04, respectively). Furthermore, increased dietary gamma-tocopherol was associated with increased 8-isoprostane concentrations (p-trend: 0.04). Engaging in any current physical activity was associated with reduced PGF2α levels (p: 0.02), as was use of multivitamins (p-trend: 0.04) and use of supplements with beta-carotene (p-trend: <0.001), vitamin C (p-trend: 0.02), vitamin E (p-trend: 0.02), iron (p-trend: 0.07), selenium (p-trend: 0.01), and zinc (p-trend: 0.01).

Physical activity in the prior 10 years was associated with reduced baseline DNA damage (p: 0.04) as was modest alcohol consumption (global p: 0.08); non-aspirin NSAID use (p-trend: < 0.001) and history of cardiovascular disease (p: 0.06) were associated with increased levels of baseline DNA damage. No factors were associated with DNA repair capacity at 15 minutes (results not shown); use of hormone replacement therapy was associated with reduced DNA repair capacity at 60 minutes (p: 0.07).

In fully-adjusted multivariate analyses, none of the specialty supplements studied were significantly associated with 8-isoprostane (Table 3). Current glucosamine use was associated with reduced PGF2α concentrations (p-trend: 0.01): persons consuming 14+ pills/week had an adjusted geometric mean

PGF2α 40% lower than non-users (225 pg/mg creatinine; 95% confidence interval (CI): 163, 312) versus 374 pg/mg creatinine; 95% CI: 326, 429). Chondroitin use was also associated with reduced PGF2α concentrations (p-trend: 0.003): persons using 14+ pills/week of chondroitin had a geometric mean PGF2α of 196 pg/mg creatinine (95% CI: 131-293), 47% lower than among non-users (371 pg/mg creatinine; 95% CI: 326-422). Because two-thirds of glucosamine users were taking glucosamine in combination with chondroitin, we additionally limited glucosamine exposure to the 19 persons using glucosamine only. We observed no association between use of glucosamine only and PGF2α (p: 0.67) (results not shown). Furthermore, use of fiber products at least once a week in the month prior was associated with 43% lower PGF2α than among non-users (208 pg/mg creatinine; 95% CI: 140-308) vs. 364 pg/mg creatinine; 95% CI: 320-413). None of the other supplements studied were associated with PGF2α concentration.

Users of co-enzyme Q10 had 58% lower baseline DNA damage than non-users (p: 0.003). The adjusted geometric mean olive tail moment was 1.02 among current users (95% CI: 0.60-1.72) compared with 2.42 among non-users (95% CI: 2.10-2.78). No other supplements were associated with baseline DNA damage. None of the supplements studied were associated with DNA repair capacity at 15 minutes (results not presented); however, use of MSM supplements was associated with reduced DNA repair capacity at 60 minutes (p: 0.002). On average, non-users had repaired 64.3% of induced DNA damage at 60 minutes (95% CI: 61.3-67.2), while users repaired an average 46.8% of induced damage at 60 minutes (95% CI: 36.7-56.8).

Furthermore, since baseline DNA damage may theoretically reflect exposure over a longer time period, we also assessed the association between supplement exposure over the 10 years prior to baseline and baseline DNA damage. The associations observed were largely similar to those observed for current exposure and are therefore not presented.

DISCUSSION

In our study, use of glucosamine, chondroitin, and fiber supplements was associated with reduced PGF2α concentrations, and use of co-enzyme Q10 supplements was associated with decreased baseline DNA damage. Use of MSM was associated with decreased DNA repair capacity at 60 minutes. None of the other supplements studied (fish oil, garlic, ginseng, ginkgo, saw palmetto) were associated with markers of oxidative stress or DNA damage.

We observed that high users of glucosamine (14+ pills/week) had 40% lower levels of PGF2α than non-users (p-trend: 0.01), and that high users of chondroitin had 48% lower levels of PGF2α than non-users (p-trend: 0.003). The percent reduction was similar or greater than was observed for use of supplements known to reduce oxidative stress, including beta-carotene, vitamin C, vitamin E and selenium (Table 2). No prior human studies have reported on glucosamine and chondroitin use and measures of oxidative stress in humans. However, results from *vitro* and animal studies support the role of glucosamine and chondroitin in reducing oxidative stress. *In vitro* work has demonstrated that sulfated glucosamine inhibits the free-radical oxidation of lipids, proteins, and DNA in a dose-dependent manner (13), while also acting to scavenge free radicals and improve the redox balance (13). Similar work has also shown that chondroitin reduces lipid peroxidation and DNA damage (15). Several but not all (56) studies have reported that glucosamine and/or chondroitin suppress IL-1β- or lipopolysaccharide-stimulated production of nitric oxide (NO) *in vitro* (57-61). This reactive nitrogen species can act to damage both lipids and DNA (13, 62-64). Animal work further supports the role of glucosamine and chondroitin in oxidative stress: in rats, glucosamine administration has been shown to inhibit superoxide generation (14), while chondroitin administration has been shown to reduce oxidative bursts of neutrophils (65) and lipid peroxidation (16, 66), while increasing total antioxidant status (65).

Furthermore, evidence from *in vitro* (30, 67), animal (68), and human (29, 31) studies suggests that glucosamine and chondroitin have anti-inflammatory effects resulting from inhibition of NFkB activity. Given that reactive species can induce inflammation via NFkB activation and that the inflammatory process can conversely generate reactive species, recent *in vitro* work has attempted to better understand the interplay between oxidative stress and inflammation as it relates to these supplements. *In vitro* studies have suggested that the mechanism by which glucosamine and chondroitin reduce NFkB activation (and the potentiation of the inflammatory cascade) may involve the inhibition of reactive oxygen species (69-71). Thus the associations observed between glucosamine and chondroitin use and PGF2α in this study could reflect either or both of these closely related biologic processes.

Since glucosamine is often, but not always, taken with chondroitin in a single daily supplement, our glucosamine and chondroitin findings may not be independent of one another. We conducted an additional exploratory analysis in which we examined the association between use of glucosamine alone and PGF2α, and found that the association between glucosamine alone and PGF2α was not statistically significant. While this may be due to limited power, it is also possible that the observed association between glucosamine use (with or without chondroitin) and PGF2α is largely driven by chondroitin or that glucosamine and chondroitin may act together, as suggested by some biologic studies (57, 72). We were unable to examine the association between chondroitin alone and PGF2a, as all chondroitin users in this study reported use of glucosamine.

We also observed that persons using fiber supplements had 43% lower PGF2α levels than non-users (p: 0.01). Limited work has been conducted on the association between fiber and oxidative stress in humans. In a cross-sectional study conducted among 246 healthy adults, fiber intake from fruits and vegetables was observed to be associated with increased total anti-oxidant capacity (73). The association between fiber and oxidative stress has also been indirectly addressed in several small human

trials, with results showing decreases in measures of lipid peroxidation (74), 8-isoprostane levels (75), and superoxide and hydrogen peroxide production (76); however, the interventions in these trials were multi-pronged (e.g., high fiber plus low –fat diet and/or exercise) and do not allow the effect of dietary fiber to be separated from the other exposures. Animal studies on the association between fiber and oxidative stress have been inconsistent, with some (77, 78), but not all (79), studies suggesting an effect. If fiber reduces oxidative stress, the mechanism has not been fully elucidated. Since increased dietary fiber intake has been associated with reduce inflammation (44), it is possible that fiber may act indirectly to reduce oxidative stress via decreased inflammation.

In our study, we also observed CoQ10 supplement users to have 60% lower levels of DNA damage than non-users (p: 0.001). Studies evaluating the association between CoQ10 and DNA damage in humans have been mixed. Two small intervention studies reported that CoQ10 supplementation decreased oxidative DNA damage (25, 26). However, other intervention studies have found no evidence of an association between CoQ10 supplementation and measures of DNA damage (20, 27, 28, 80), possibly due to small sample size or use of doses insufficient to yield biologic effect (21). Despite the inconsistent human studies, *in vitro* research suggests that CoQ10 may reduce DNA damage, as measured in healthy human nasal tissue (17) and human lymphocytes (18).

If CoQ10 does act to reduce DNA damage, it is likely through a reduction in oxidative stress (25). CoQ10 is an essential cofactor in the electron transport chain, and is a well-known intracellular antioxidant (19, 22, 81). As a redox molecule, CoQ10 has both oxidized and reduced forms, and acts to stabilize free radicals (82). However, human research is not consistent regarding the effect of CoQ10 on lipid peroxidation: while our present study and two other small studies reported no association between CoQ10 supplementation and markers of oxidative stress (20, 21), three other larger trials have observed a significant association between CoQ10 supplementation and measures of oxidative stress (22-24).

We also observed use of MSM to be associated with reduced DNA repair capacity at 60 minutes. No other human research has reported on the association between MSM use and DNA repair capacity. While limited, some *in vitro* (36), animal (83) and human research (84) suggests that MSM may reduce oxidative stress. Since reduced oxidative stress would be expected to *improve*, not diminish, DNA repair capacity (49), there is little support for our finding.

Results of this study suggest that glucosamine and chondroitin supplements may be associated with reduced oxidative stress, providing a biologic mechanism to support studies which have shown glucosamine and chondroitin to be association with reduced risk of CRC (85), lung cancer (85, 86), and total mortality (87, 88). We also observed fiber supplement use to be associated with reduced oxidative stress, adding support to prior epidemiologic which suggest that fiber might reduce risk of and cardiovascular disease (89), and various cancers, including colorectal cancer (90-92). Further research is needed to better understand these associations and how use of these supplements might affect disease risk.

This study has several strengths. First, all supplements except fiber were ascertained by supplement inventory and interview at the participants home, at which time the ingredients contained within each supplement were transcribed. Transcribing the ingredients from supplement bottles likely increased the accuracy of exposure measurement as compared to self-report. Furthermore, in order to reduce measurement error for dietary intake, self-reported BMI, and self-reported smoking, we averaged values from the baseline and biomarker study questionnaires, which may reduce concerns of potential residual confounding due to measurement error in these variables. Another advantage of this study is that our population oversampled supplement users, and the majority of non-users of any given supplement studied were likely using other supplements. The use of a comparison group largely

comprised of supplement users may reduce concern regarding potential residual confounding by healthy behaviors associated with supplement use.

This study is not without limitation. First, our study was conducted in a relatively small population, potentially limiting our power to detect association for the less-commonly used supplements. Our ability to detect associations may have also been limited by the fact that validity study participants were instructed not to use the supplement on the day of interview/specimen collection. This may have been a particular concern for supplements with a shorter half-life, though it should be noted that the majority of supplement users did report use within the day prior. Furthermore, in our analyses of DNA damage and DNA repair capacity, we made additional exclusions (such as exclusion of those with prior cancer, those with non-viable leukocytes), further limiting our sample size (and power), potentially explaining the small number of factors associated with these outcomes. The generalizability of these study findings may be limited by the over-representation of supplement users; furthermore, despite use of a more conservative alpha in assessing associations between supplement use and oxidative stress/DNA damage, it is we cannot rule out the presence of false positives.

Another limitation of the interpretation of our results is lack of consistency across biomarkers of oxidative stress and DNA damage. It is unclear why the factors associated with the two biomarkers of oxidative stress, PGF2α and 8-isoprostane, differed in our study and why these two biomarkers were only modestly correlated with one another. This may be due to differences in biologic pathways reflected, sensitivity of biomarkers, or measurement error (6, 93). In our study, we observed modest intraclass correlation coefficients (ICCs) when assessing the between-plate reliability of 8-isoprostane and PGF2a on 22 duplicate-pairs. The ICC for PGF2α was 0.80 for the log-transformed measure itself, and 0.32 when divided by creatinine, while the ICC for 8-isoprostane was 0.85, which reduced to 0.46 when divided by creatinine. However, it should be noted that in our study, several expected predictors

of oxidative stress were associated with our measures of 8-isoprostane and PGF2α. For example, supplements known to be associated with reduced oxidative stress, such as beta-carotene, vitamin C, vitamin E, and selenium supplements, were all significantly associated with reduced PGF2α in our study, while smoking, supplementary beta-carotene and supplementary alpha-tocopherol were associated with 8-isoprostane. It is also unclear why factors associated with oxidative stress were not reflected in measures of DNA damage. To this end, we observed no correlation between measures of oxidative stress and measures of DNA damage/DNA repair capacity, and others have reported only modest correlation between 8-isoprostane and oxidative DNA damage (94). It may be that factors associated with oxidative stress do not translate to measures of DNA damage (such as DNA strand breakage), as DNA damage may result from factors beyond oxidative stress. Therefore, the relative contribution of any one factor (such as oxidative stress) may be too small to see any effect on DNA damage. However, any measurement error (in any of our assays) would be expected to be non-differential, likely attenuating results toward the null.

In summary, results suggest that glucosamine, chondroitin, and fiber supplements are associated with reduced oxidative stress in humans. However, as noted previously, it is difficult to disentangle the observed associations between these supplements and oxidative stress from possible anti-inflammatory effects, as the two biologic processes are closely intertwined. We also observed CoQ10 supplementation to be associated with reduced levels of DNA damage. Further research is needed to better understand the association between use of these supplements and oxidative stress/DNA damage, as oxidative stress and DNA damage have been implicated in several diseases. Our results provide evidence a potential mechanism by which these supplements may affect disease risk, warranting further research on these potential preventives.

Table 1. Correlation Matrix: biomarkers of oxidative stress, DNA damage, and DNA repair capacity

	8-isoprostane pg/mg creatinine	PGF2α pg/mg creatinine	Baseline DNA Damage olive tail moment	DNA Repair Capacity 15 Min. olive tail moment (%)	DNA Repair Capacity 60 Min. olive tail moment (%)
8-isoprostane pg/mg creatinine	1.00				
PGF2α pg/mg creatinine	0.22 *(P<0.001)*	1.00			
Baseline DNA Damage olive tail moment	-0.05 *(P=0.61)*	-0.04 *(P=0.63)*	1.00		
DNA Repair Capacity 15 Min. olive tail moment (%)	0.03 *(P=0.77)*	0.15 *(P=0.13)*	-0.30 *(P=0.003)*	1.00	
DNA Repair Capacity 60 Min. olive tail moment (%)	-0.03 *(P=0.79)*	0.15 *(P=0.14)*	-0.27 *(P=0.006)*	0.3506 *(P<0.001)*	1.00

Table 2. Demographic and Lifestyle Factors and Their Associations with Measures of Oxidative Stress and DNA Damage within the VITAL Biomarker Study

		8-isoprostane pg/mg creatinine (n=206)		PGF2α pg/mg creatinine (n=206)		Baseline DNA Damage olive tail moment (n=119)		DNA Repair Capacity 60 Min. olive tail moment (%) (n=97)	
	N (%)	Adjusted geometric mean[a]	95% CI	Adjusted geometric mean[a]	95% CI	Adjusted geometric mean[a]	95% CI	Adjusted mean[a]	95% CI
Demographic									
Age									
50-<55	53 (24.1)	779	649, 935	337	260, 436	2.11	1.59, 2.81	65.5	59.8, 71.3
55-<60	55 (25.0)	842	708, 1002	376	295, 480	2.36	1.75, 3.19	61.4	55.8, 67.1
60-<65	37 (16.8)	750	607, 926	322	239, 434	3.08	2.05, 4.61	55.7	47.3, 64.0
65-<70	30 (13.6)	691	545, 875	376	269, 525	2.22	1.48, 3.33	67.3	59.5, 75.1
70+	45 (20.5)	825	672, 1012	310	232, 413	2.02	1.46, 2.8	61.0	54.3, 67.6
		P (continuous): 0.75		*P (continuous): 0.44*		*P (continuous): 0.73*		*P (continuous):0.21*	
Gender									
Female	108 (49.1)	879	775, 998	362	303, 433	2.37	1.90, 2.96	64.3	59.8, 68.8
Male	112 (50.9)	705	623, 797	327	275, 389	2.21	1.80, 2.70	61.2	57.2, 65.2
		P: 0.02		*P: 0.42*		*P: 0.65*		*P: 0.32*	
Race/ethnicity									
Non-white	208 (94.6)	783	715, 857	344	303, 391	2.28	1.96, 2.66	62.9	59.9, 65.9
White	12 (5.5)	822	570, 1186	337	201, 564	2.2	1.13, 4.29	54.7	40.0, 69.3
		P: 0.80		*P: 0.94*		*P: 0.92*		*P: 0.28*	
Education									
HS Grad/GED or Less	27 (12.3)	1131	870, 1471	416	284, 608	2.02	1.26, 3.22	63.6	54.3, 72.9
Some College/Tech	76 (34.6)	862	746, 997	319	259, 394	2.54	1.96, 3.29	59.7	54.4, 64.9
College Grad	59 (26.8)	675	571, 797	349	274, 445	1.99	1.51, 2.63	62.7	57.2, 68.1
Advanced Degree	58 (26.4)	692	584, 818	344	270, 439	2.42	1.8, 3.26	65.7	59.7, 71.6
		P-trend: 0.001		*P-trend: 0.81*		*P-trend: 0.93*		*P-trend: 0.31*	
Lifestyle/Anthropometric									
Smoking (pack-years)									
Never smokers	111 (50.5)	711	628, 805	332	279, 396	2.31	1.87, 2.86	63.4	59.1, 67.7
Below Median (<18)	55 (25.0)	728	610, 869	316	246, 405	2.03	1.53, 2.69	63.2	57.7, 68.7
Above Median (18+)	54 (24.6)	1034	867, 1234	400	312, 513	2.55	1.87, 3.48	59.6	53.0, 66.2
		P-trend: 0.002		*P-trend: 0.30*		*P-trend: 0.75*		*P-trend: 0.40*	
Alcohol (grams/week)									
Lowest tertile (<3)	73 (33.3)	777	666, 906	336	271, 417	2.72	2.08, 3.55	59.7	54.2, 65.3
Mid-tertile (3-<56)	73 (33.3)	760	650, 889	388	312, 483	1.81	1.39, 2.35	66.2	61.0, 71.3
Highest tertile (56+)	73 (33.3)	816	700, 949	311	252, 385	2.40	1.88, 3.05	61.6	56.6, 66.6
		P (overall): 0.81		*P (overall): 0.34*		*P (overall): 0.08*		*P (overall): 0.68*	

BMI (kg/m^2)									
<25	83 (37.7)	744	642, 862	336	273, 415	2.14	1.64, 2.79	65.1	59.6, 70.5
25-<30	100 (45.5)	763	671, 868	337	281, 404	2.47	1.99, 3.06	59.6	55.3, 63.9
30+	37 (16.8)	965	775, 1201	383	281, 522	2.04	1.42, 2.93	66.3	59.2, 73.4
		P-trend: 0.09		*P-trend: 0.57*		*P-trend: 1.00*		*P-trend: 0.99*	
Current Physical Activity (moderate/vigorous)									
None	121 (55.5)	831	739, 936	393	334, 464	2.32	1.91, 2.83	61.5	57.5, 65.4
Any	97 (44.5)	722	631, 827	288	239, 348	2.21	1.76, 2.78	64.1	59.3, 68.9
		P: 0.13		*P: 0.02*		*P: 0.76*		*P: 0.42*	
10-yr Physical Activity (moderate/vigorous)									
None	84 (38.7)	845	733, 975	366	302, 444	2.80	2.21, 3.54	60.8	55.9, 65.6
Any	133 (61.3)	749	668, 840	340	291, 397	2.03	1.69, 2.45	64.0	60.2, 67.8
		P: 0.20		*P: 0.56*		*P: 0.04*		*P: 0.30*	
Medication use									
Regular-Dose Aspirin									
No	161 (73.9)	773	697, 857	348	301, 403	2.28	1.91, 2.72	61.2	57.7, 64.7
Low (1-3 days/wk)	24 (11.0)	836	645, 1082	283	197, 408	1.76	1.18, 2.63	68.8	61, 76.6
High (4+ days/wk)	33 (15.1)	817	649, 1029	372	269, 515	2.82	1.94, 4.09	63.1	55.1, 71.1
		P-trend: 0.58		*P-trend: 0.98*		*P-trend: 0.57*		*P-trend: 0.34*	
Non-aspirin NSAID									
No	132 (61.1)	759	676, 851	357	304, 419	1.89	1.58, 2.26	61.7	58.1, 65.3
Low (1-3 days/wk)	45 (20.8)	870	714, 1061	371	281, 491	2.56	1.88, 3.49	65.2	58.4, 72.0
High (4+ days/wk)	39 (18.1)	802	654, 983	279	210, 372	3.97	2.8, 5.61	63.2	54.2, 72.1
		P-trend: 0.46		*P-trend: 0.22*		*P-trend: <0.001*		*P-trend: 0.54*	
HRT									
No	159 (73.6)	790	707, 884	358	306, 420	2.38	1.92, 2.96	66.0	61.5, 70.5
Yes	57 (26.4)	789	642, 969	311	231, 417	2.14	1.48, 3.1	56.1	48.4, 63.8
		P: 0.99		*P: 0.45*		*P: 0.67*		*P: 0.07*	
Medical history									
History of CVD									
No	190 (86.4)	773	703, 851	335	293, 383	2.16	1.85, 2.52	62.5	59.4, 65.7
Yes	30 (13.6)	862	670, 1111	401	281, 572	3.53	2.19, 5.69	62.9	52.1, 73.8
		P: 0.44		*P: 0.36*		*P: 0.06*		*P: 0.95*	
History of Cancer									
No	180 (81.8)	802	729, 883	347	303, 398	N/A	N/A	N/A	N/A
Yes	40 (18.2)	702	565, 872	325	240, 441				
		P: 0.27		*P: 0.70*					
History of Diabetes									
No	213 (96.8)	795	728, 869	340	300, 385	2.25	1.94, 2.62	62.4	59.3, 65.4
Yes	7 (3.2)	538	331, 875	467	235, 927	3.57	1.38, 9.23	69.4	52.1, 86.7
		P: 0.12		*P: 0.37*		*P: 0.35*		*P: 0.44*	

Supplemental nutrients[c]										
Multivitamin										
No	82 (37.4)	803	696, 926	442	356, 550	2.29	1.81, 2.89	64.9	60.1, 69.6
Yes	137 (62.6)	778	695, 871	335	267, 421	2.27	1.87, 2.76	61.0	57.2, 64.9
		P: 0.74		*P: 0.04*		*P: 0.95*		*P: 0.23*	
Beta-Carotene (µg/d)									
None	86 (39.3)	883	769, 1015	450	372, 545	2.36	1.86, 2.99	62.6	57.8, 67.3
Low (≤600)	42 (19.2)	720	587, 884	319	240, 423	2.33	1.65, 3.3	64.0	56.8, 71.2
High (>600)	91 (41.6)	734	640, 841	276	229, 333	2.18	1.72, 2.75	62.0	57.2, 66.7
		P-trend: 0.06		*P-trend: <0.001*		*P-trend: 0.63*		*P-trend: 0.86*	
Vitamin C (mg/d)									
None	47 (21.5)	938	778, 1132	487	375, 632	2.52	1.8, 3.52	63.8	56.9, 70.6
Low (≤560)	88 (40.2)	735	639, 845	315	259, 382	2.11	1.67, 2.65	61.0	56.4, 65.6
High (>560)	84 (38.4)	766	663, 884	311	254, 379	2.36	1.84, 3.04	64.1	58.8, 69.4
		P-trend: 0.15		*P-trend: 0.02*		*P-trend: 0.90*		*P-trend: 0.82*	
Vitamin E (mg/d α-tocopherol)									
None	42 (19.2)	1017	834, 1239	550	418, 724	2.06	1.45, 2.94	63.9	57, 70.8
Low (≤430)	93 (42.5)	732	642, 835	290	242, 348	2.48	1.99, 3.11	61.1	56.6, 65.6
High (>430)	84 (38.4)	750	649, 867	330	270, 404	2.15	1.67, 2.76	63.7	58.6, 68.9
		P-trend: 0.04		*P-trend: 0.02*		*P-trend: 0.94*		*P-trend: 0.88*	
Iron (mg/day)									
None	123 (56.2)	836	742, 942	442	356, 550	2.55	1.72, 2.55	63.6	59.7, 67.6
Low (≤18)	77 (35.2)	715	618, 827	335	267, 421	3.24	1.98, 3.24	60.7	55.7, 65.7
High (>18)	19 (8.7)	811	602, 1093	289	237, 351	4.46	1.51, 4.46	63.8	50.6, 77
		P-trend: 0.31		*P-trend: 0.07*		*P-trend: 0.23*		*P-trend: 0.54*	
Selenium (µg/day)									
None	74 (33.8)	857	737, 997	448	364, 552	2.21	1.72, 2.86	62.7	57.6, 67.9
Low (≤20)	54 (24.7)	716	598, 857	309	241, 396	2.35	1.73, 3.17	64.3	58.4, 70.2
High (>20)	91 (41.6)	776	677, 891	296	245, 358	2.29	1.8, 2.91	61.2	56.3, 66.1
		P-trend: 0.37		*P-trend: 0.01*		*P-trend: 0.86*		*P-trend: 0.66*	
Zinc (mg/day)									
None	67 (30.6)	899	769, 1051	442	356, 550	2.35	1.78, 3.09	62.2	56.5, 67.9
Low (≤15)	64 (29.2)	667	567, 784	335	267, 421	2.25	1.71, 2.96	61.9	56.5, 67.4
High (>15)	88 (40.2)	802	698, 922	289	237, 351	2.25	1.76, 2.86	63.4	58.5, 68.3
		P-trend: 0.36		*P-trend: 0.01*		*P-trend: 0.82*		*P-trend: 0.73*	
Dietary factors									
Dietary γ-Tocopherol (mg/d)[b]									
Q1 (<9.5)	55 (25.4)	693	557, 863	331	241, 454	2.56	1.68, 3.90	68.6	59.9, 77.3
Q2 (9.5- <13.1)	54 (24.9)	835	694, 1005	411	315, 537	2.53	1.80, 3.54	63.1	55.6, 70.6
Q3 (13.1-<17.1)	54 (24.9)	669	562, 796	325	253, 418	2.06	1.54, 2.74	64.2	58.4, 69.9
Q4 (17.1+)	54 (24.9)	975	775, 1226	320	230, 446	2.11	1.41, 3.16	56.3	48.3, 64.2
		P (continuous): 0.04		*P (continuous): 0.92*		*P (continuous): 0.27*		*P (continuous): 0.26*	

Abbreviations: 95% CI (95% confidence interval); BMI (body mass index); CVD (cardiovascular disease); HRT (hormone replacement therapy); NSAID (non-steroidal anti-inflammatory drug)

[a] Means are presented for untransformed variables (DNA repair capacity); geometric means are presented for variables which have been ln-transformed to normalize their distributions (baseline DNA damage; 8-isoprostane; PGF2α); all means and geometric means are adjusted for age (continuous), sex, and packyears smoked (ordinal: none, below the median, above the median)

[b] Further adjusted for energy intake (continuous)

[c] From multivitamins and individual supplements

Table 3. Association Between Current Specialty Supplement Use And Measures of Oxidative Stress and DNA Damage

Supplement	N (%)	8-isoprostane[a] pg/mg creatinine (n=206)		PGF2α[b] pg/mg creatinine (n=206)		Baseline DNA Damage[c] olive tail moment (n=119)		DNA Repair Capacity 60 Min.[d] olive tail moment (%) (n=97)	
		Adjusted Geometric Mean[e]	95% CI	Adjusted Geometric Mean[e]	95% CI	Adjusted Geometric Mean[e]	95% CI	Adjusted Mean[e]	95% CI
Glucosamine[f]									
No Use	167 (75.9)	779	705, 859	374	326, 429	2.32	1.97, 2.74	62.6	59.1, 66.1
Low (<14 pills/wk)	21 (9.6)	788	581, 1070	334	220, 507	3.56	2.20, 5.76	58.6	49.5, 67.7
High (14+ pills/wk)	32 (14.6)	822	650, 1039	225	163, 312	1.79	1.25, 2.57	67.1	59.3, 74.8
		P-trend: 0.84		*P-trend: 0.01*		*P-trend: 0.42*		*P-trend: 0.47*	
Chondroitin[f]									
No Use	186 (84.6)	797	725, 875	371	326, 422	2.23	1.90, 2.62	62.6	59.4, 65.7
Low (<14 pills/wk)	15 (6.8)	697	492, 989	294	177, 489	3.30	1.93, 5.65	55.4	45.1, 65.6
High (14+ pills/wk)	19 (8.6)	749	563, 998	196	131, 293	2.37	1.52, 3.68	72.4	62.4, 82.5
		P-trend: 0.55		*P-trend: 0.003*		*P-trend: 0.52*		*P-trend: 0.26*	
Fish Oil[g]									
No Use	193 (87.7)	772	704, 847	339	297, 386	2.32	2.01, 2.69	62.9	59.9, 66
Use	27 (12.3)	891	683, 1163	390	273, 559	2.25	1.35, 3.77	61.1	50.1, 72
		P: 0.33		*P: 0.47*		*P: 0.91*		*P: 0.75*	
Co-enzyme Q10[h]									
No Use	197 (89.6)	791	722, 867	344	302, 391	2.42	2.10, 2.78	62.5	59.4, 65.5
Use	23 (10.5)	737	557, 976	349	234, 523	1.02	0.60, 1.72	66.5	55.5, 77.6
		P: 0.64		*P: 0.94*		*P: 0.003*		*P: 0.49*	
MSM[f]									
No Use	199 (90.5)	795	726, 870	354	311, 402	2.32	2.00, 2.68	64.3	61.3, 67.2
Use	21 (9.6)	700	524, 936	267	178, 401	2.29	1.31, 4.03	46.8	36.7, 56.8
		P: 0.42		*P: 0.20*		*P: 0.97*		*P: 0.002*	
Garlic									
No Use	195 (88.6)	800	731, 876	342	301, 389	2.42	2.09, 2.79	62.9	59.8, 66.0
Use	25 (11.4)	677	519, 884	364	252, 526	1.49	0.92, 2.41	61.4	51.2, 71.6
		P: 0.25		*P: 0.75*		*P: 0.06*		*P: 0.79*	
Ginseng[i]									
No Use	202 (91.8)	773	707, 846	342	301, 388	2.36	2.03, 2.73	62.9	59.9, 66.0
Use	18 (8.2)	928	694, 1241	372	243, 569	2.01	1.28, 3.15	61.1	50.9, 71.3
		P: 0.24		*P: 0.71*		*P: 0.51*		*P: 0.74*	
Ginkgo[i]									
No Use	196 (89.1)	792	722, 868	346	305, 394	2.39	2.06, 2.77	63.5	60.5, 66.5
Use	24 (10.9)	740	569, 961	329	224, 484	1.77	1.14, 2.76	56.1	46.5, 65.6
		P: 0.63		*P: 0.81*		*P: 0.22*		*0.15*	
Saw Palmetto[j]									
No Use	97 (86.6)	708	622, 806	330	272, 400	2.38	1.95, 2.89	60.7	56.6, 64.7
Use	15 (13.4)	644	461, 898	317	192, 522	1.43	0.83, 2.46	66.6	56.2, 77.1
		P: 0.61		*P: 0.88*		*P: 0.10*		*P: 0.30*	

Fiber[k]									
No Use	196 (89.9)	769	703, 842	364	320, 413	2.35	2.03, 2.73	62.5	59.4, 65.6
Use	22 (10.1)	937	709,1238	208	140, 308	2.00	1.24, 3.22	65.0	55.7, 74.3
		P: 0.19		*P: 0.01*		*P: 0.52*		*P: 0.63*	

Abbreviations: 95% CI (95% confidence interval); MSM (methylsulfonylmethane)

[a] Analyses of 8-isoprostane adjusted for age (continuous), gender, education (ordinal: HS grad/GED or less, some college/tech college grad, advanced degree), packyears-smoked (ordinal: none, below median, above median), body mass index (ordinal: tertiles), supplementary beta-carotene (ordinal: none, low, high), supplementary alpha-tocopherol (ordinal: none, low, high), dietary gamma-tocopherol (continuous), energy intake (continuous

[b] Analyses of PGF2α adjusted for age (continuous), gender, packyears-smoked (ordinal: none, below median, above median), any moderate or vigorous physical activity (no/yes), current multivitamin use (no/yes), supplementary beta-carotene (ordinal: none, low, high), supplementary vitamin C (ordinal: none, low, high), supplementary alpha-tocopherol (ordinal: none, low, high), supplementary selenium (ordinal: none, low, high), supplementary iron (ordinal: none, low, high), supplementary zinc (ordinal: none, low, high)

[c] Analyses of baseline DNA damage adjusted for age (continuous), gender, packyears-smoked (ordinal: none, below median, above median), alcohol consumption (categorical: tertiles), any moderate or vigorous physical activity over 10-years prior to baseline (no/yes), current use of non-aspirin non-steroidal anti-inflammatory drugs (ordinal: none, low, high)

[d] Analyses of DNA repair capacity at 60 minutes adjusted for age (continuous), gender, packyears-smoked (ordinal: none, below median, above median), current HRT (no/yes)

[e] Means are presented for untransformed variables (DNA repair capacity); geometric means are presented for variables which have been ln-transformed to normalize their distributions (baseline DNA damage; 8-isoprostane; PGF2α)

[f] Analyses of glucosamine, chondroitin and MSM additionally adjusted for the indication of arthritis or chronic pain

[g] Analyses of fish oil additionally adjusted for indications of cardiovascular disease and memory loss

[h] Analyses of co-enzyme Q10 were additionally adjusted for the indication of cardiovascular disease

[i] Analyses of ginseng and ginkgo additionally adjusted for the indication of memory loss

[j] Analyses of saw palmetto additionally adjusted for the indication of benign prostatic hyperplasia; analyses of saw palmetto were limited to men

[k] Analyses of fiber additionally adjusted for the indication of constipation

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Chapter 4. Association of specialty supplement use with C-reactive protein

ABSTRACT

Laboratory evidence suggests that certain specialty supplements have anti-inflammatory properties, though evidence in humans remains limited. A nationally representative sample of 9947 adults from the 1999-2004 cycles of the National Health and Nutrition Examination Survey was used to assess the associations between specialty supplement use and inflammation, as measured by serum high-sensitivity c-reactive protein (hs-CRP). Using survey-weighted multivariate linear regression, significant reductions in hs-CRP concentrations were associated with regular use of glucosamine (17%; 95% CI: 7%, 26%), chondroitin (22%; 95% CI: 8%, 33%), and fish oil (16%; 95% CI: 0.3%, 29%). No associations were observed between concentration of hs-CRP and regular use of methylsulfonylmethane (MSM), garlic, ginkgo biloba, saw palmetto, or pycnogenol-containing supplements. These results suggest that glucosamine and chondroitin supplements are associated with reduced inflammation in humans and provide further evidence to support an inverse association between fish oil supplement use and inflammation. It is important to further investigate the potential anti-inflammatory role of these supplements, as there is need to identify safe and effective ways to reduce inflammation and the burden of inflammation-related diseases such as cancer and cardiovascular disease.

INTRODUCTION

Inflammation has been implicated in the etiology of several chronic diseases, including cardiovascular disease and several cancers (1-4). Consistent with these observations, the anti-inflammatory drug, aspirin, has been found to reduce risk of cardiovascular disease (5, 6) and colorectal cancer (7) in randomized control trials (RCTs), and has been associated with reduced risk of other cancers in observational studies (8-10). Concerns remain about the adverse effects of long-term use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (6, 11-13); consequently, there is need to identify other safe and effective measures to reduce inflammation and inflammation-related diseases.

Laboratory studies suggest that certain non-vitamin, non-mineral "specialty" supplements may act to reduce inflammation. These include glucosamine (14-19), chondroitin (20, 21), methylsulfonylmethane (MSM) (22), omega-3 polyunsaturated fatty acid (PUFA) containing fish oil supplements (23, 24), garlic (25-27), ginseng (28, 29), ginkgo biloba (30), saw palmetto (31), and pycnogenol-containing supplements (32, 33). Despite the suggested anti-inflammatory properties of these supplements, evidence in humans remains limited. Of these supplements, omega-3 PUFA supplementation has been the most well-studied, with recent RCT evidence suggesting that omega-3 supplements reduce inflammation (34, 35).

Given the current gap in our knowledge about the biologic effects of these supplements and the need for safe and effective measures to reduce inflammation, study of these supplements is warranted. We used data collected by the National Health and Nutrition Examination Survey (NHANES) to assess whether the aforementioned supplements are associated with inflammation in US adults, with inflammation measured by serum high sensitivity c-reactive protein (hs-CRP).

METHODS

Data source/ study population

The analyses were based on data collected as part of the 1999-2000, 2001-2002, and 2003-2004 cycles of NHANES, a nationally representative cross-sectional survey of civilian, non-institutionalized persons living in the United States (36). These cycles were selected because they included data on the exposures (supplements), outcome (hs-CRP), and covariates of interest.

Information on health and health-behaviors was collected at home interview, with further data, physical examination, and laboratory tests collected from a subset of participants at Mobile Examination Centers. This stratified complex multi-stage probability based survey over-samples persons aged 60+, low-income individuals, as well as persons of certain racial/ethnic groups. All participants are assigned weights to account for unequal sampling probability.

Of the 12,063 persons aged 25 and older for whom hs-CRP was measured, we further excluded 198 persons with outlying CRP values (those with CRP values in the top 2% for their age group, gender, and body mass index (BMI) category). Such an approach was used in order to exclude persons with acute illness, as the definition of outlying values may vary across factors such as age, gender, and BMI (37). For example, among underweight and normal weight men aged 25-39, the 98th percentile was 10.7 mg/L, while the 98th percentile was 30.3 mg/L for severely obese men aged 60 and older. Corresponding 98th percentiles were higher among women (18.5mg/L and 38.3 mg/L, respectively). We further excluded women between the ages of 25 and 59 with positive or unknown pregnancy test result (n=524), as well as those participants with missing dietary data or who failed dietary quality control checks (n= 963, described below), or who had missing information on the other covariates or exposures of interest: educational status (n=25), smoking status (n=19), measured height/weight (n=356), physical activity (n=11), aspirin/NSAID use (n=28), statin use (n=20), diabetes history (n=4), history of coronary heart

disease, angina or myocardial infarction (n=63), joint pain or arthritis (n=117), memory loss/confusion (n=10), or whether any supplements were used in the last 30 days (n=18). The above-listed exclusions are not mutually exclusive, as some were excluded for more than one reason. After making theses exclusions, 9947 participants remained for analysis.

All participants provided informed consent and the survey was approved by the National Center for Health Statistics Institutional Review Board. NHANES data are publicly available and are considered exempt by the University of Washington Institutional Review Board.

Supplement use

The NHANES interview included a series of questions related to supplement use. Participants who indicated that supplements were used in the 30 days prior to interview were asked to list all supplements used in this period and to provide information on use of each supplement, including usual frequency of use. Information on each reported supplement was then linked to a database containing information on ingredients contained within each supplement type, which was then used to identify individual supplements and supplement combinations containing the ingredients of interest. We abstracted information on use of specialty supplements hypothesized to reduce inflammation, including: glucosamine, chondroitin, methylsulfonylmethane (MSM), fish oil, garlic, ginseng, pycnogenol-containing supplements (Grapeseed extract, Pine Bark), ginkgo, and saw palmetto. Regular use (yes/no) of a given supplement was defined as use of a supplement during the month prior to baseline as well as usual frequency of at least 20 days per month. Persons reporting no use were considered “non-users” and those reporting usual use on less than 20 days per month were excluded from supplement-specific analyses, as were persons missing information on usual frequency of use.

Outcome (hsCRP)

CRP, an acute phase protein synthesized as a result of inflammation, was used as a measure of inflammation in this study. Serum hs-CRP was measured by latex-enhanced nephelometry (38), with reported values ranging from 0.1 mg/L to 50.5 mg/L. The lower detectable limit of this hs-CRP assay was 0.2 mg/L; values below this lower detectable limit were assigned a value of 0.1 mg/L by NHANES. To normalize the right-skewed distribution, hs-CRP was log-transformed and all analyses used these log-transformed values as a continuous measure of inflammation. Values have been exponentiated for presentation.

Covariates

Covariates for adjustment were selected *a priori* based on associations with CRP in prior studies (39-53). All adjusted models included age (25-29, 30-39, 40-49, 50-59, 60-69, 70+) and gender.

Multivariate analyses were additionally adjusted for race/ethnicity (non-Hispanic white, Mexican American, other Hispanic, non-Hispanic Black, mixed race/other), education (less than high school, high school graduate or equivalent, some college/associates degree, college graduate or above), cigarette smoking history (current, former, or never smoker), and BMI (with weight and height measured at interview). BMI (kg/m^2) was categorized as follows: <18.5 (underweight), 18.5-<25 (normal weight), 25-<30 (overweight), 30-<35 (obese), and ≥35 (severely obese).

We also adjusted for leisure time physical activity (LTPA). Among those who reported engaging in moderate or vigorous LTPA in the last month, we calculated the MET-minutes per reported activity, after which we summed the MET-minutes per person across all reported activities. This variable is presented as average MET-minutes per week of LTPA and was categorized into three groups (no reported LTPA, <600 MET-minutes per week of LTPA, and ≥600 MET-minutes per week of LTPA).

All fully adjusted models additionally included use of vitamin E supplements and 3 dietary variables (dietary fiber, fat, and total energy intake), with dietary intake determined by 1 or 2 day recall (second recall included where available). Each recall ascertained dietary intake in the 24-hour period prior to dietary interview (midnight to midnight) and was collected at the time of examination or by telephone after examination. Approximately 32% of the study population had a second day of reliable recall collected: this second day of recall was collected only for the 2003-2004 cycle and was collected at least 3 days after the initial recall, with the number of days between recalls variable (36). For those with a second reliable day of recall, we averaged intake over the two recalls to better estimate usual intake. If a given dietary recall was deemed unreliable according to NHANES criteria, data from the recall were unavailable and therefore excluded. We further excluded men reporting average energy intake <800 or >5000 kcal per day, as well as women reporting average energy intake of <600 or >4000 kcal/day. Dietary factors were categorized into quintiles based on distribution of raw numbers in final dataset.

We also adjusted for current aspirin use and current non-aspirin NSAID use (yes/no, both defined as daily or nearly daily use in the last 30 days), as well as current statin use (yes/no, ascertained from a database of current medications). Adjustment was also made for history of medical conditions associated with CRP levels, including diagnosis of diabetes (yes, no, or borderline; excludes gestational diabetes) and history of heart disease (diagnosis of coronary heart disease, angina, or myocardial infarction by a health professional). Finally, where available, we adjusted for the main indications of supplement use. Supplements for which joint pain/arthritis is considered an indication of use (glucosamine, chondroitin, MSM, fish oil) were additionally adjusted for joint pain/arthritis, defined as report of doctor-diagnosed arthritis or report of joint pain not caused by injury. Analyses of supplements indicated for memory loss (fish oil, ginkgo) were further adjusted for self-reported memory loss/confusion.

Statistical analysis

Linear regression was used to model the association between regular use of each supplement and log-transformed hs-CRP, adjusted for covariates:

$$\ln(\text{hs-CRP}) = \alpha + \beta 1 \cdot X1 + \beta 2 \cdot X2 + \ldots$$

where *X1* and *X2* , etc are indicator variables for each category of the independent variables. We present the results as e^{β}, which represents the ratio of geometric mean hs-CRP among those in the category of interest to those in the reference category (e.g., ratio of hs-CRP among regular glucosamine users to hs-CRP among non-users). Analyses were adjusted for age group and gender in an initial model and multivariate adjusted for the factors previously described in a fully adjusted model. We considered additional adjustment for alcohol consumption, as well as substitution of waist circumference for BMI and saturated fat intake for total fat intake. Inclusion of these variables did not materially change the observed associations between specialty supplement use and hs-CRP; therefore, results from this alternative model are not presented. We also conducted stratified analyses to assess whether the associations between regular supplement use and hs-CRP vary by gender. Tests for multiplicative interaction between supplement use and gender in an unstratified model were conducted, with statistical significance of resulting 2-sided P-values assessed at the alpha=0.05 level.

Due to the stratified multi-stage sampling design of the NHANES data, analyses were weighted to reflect sampling probabilities, so as to allow for representation of the US population. All statistical analyses were conducted using Stata version 11 software (StataCorp IC, College Station, TX).

RESULTS

As shown in Table 1, hs-CRP is positively associated with increasing age and BMI: in multivariate adjusted models, those with BMI over 35 kg/m^2 have a geometric mean hs-CRP of 4.85 mg/L, while persons with BMI less than 18.5 kg/m^2 have a geometric mean hs-CRP of 0.73 mg/L. Hs-CRP levels are inversely associated with education and physical activity. Furthermore, women have higher hs-CRP than men, current smokers have higher hs-CRP than non-smokers, and those with history of heart disease have higher hs-CRP than those without heart disease. Increasing dietary fiber intake is associated with decreased hs-CRP levels, and statin use is associated with lower hs-CRP. However, vitamin E supplement use, dietary energy intake, dietary fat intake, diabetes, aspirin use, non-aspirin NSAID use, arthritis/joint pain, and memory loss do not appear to be associated with hs-CRP levels in the multivariate-adjusted estimates.

Table 2 presents the associations of regular use (20+ days/month) of specialty supplements with CRP levels. The weighted percent of regular use ranges from 1.2% for MSM use to 4.4% for ginseng use. In the fully adjusted model, regular use of glucosamine is associated with a statistically significant 17% reduction in hs-CRP (as compared to non-use) (Ratio: 0.83, 95% CI: 0.74, 0.93) and chondroitin with a 22% reduction in hs-CRP (Ratio: 0.78, 95% CI: 0.67, 0.92). Regular fish oil use is also associated with a significant 16% reduction in hs-CRP (Ratio: 0.84: 95% CI: 0.71, 0.997). Use of any of the remaining supplements (MSM, garlic, ginseng, ginkgo, saw palmetto, and pycnogenol-containing supplements) is not statistically significantly associated with hs-CRP.

Furthermore, we observe significant interactions by gender for the associations of glucosamine use with hs-CRP (*P*-interaction=0.05), and chondroitin use with hs-CRP (*P*-interaction=0.03). Among women, regular glucosamine use is associated with a 27% reduction in hs-CRP (Ratio: 0.73, 95% CI: 0.61, 0.88), and regular chondroitin use is associated with a 33% reduction in hs-CRP (Ratio: 0.67, 95% CI: 0.53,

0.84), while the associations among men are small and non-significant. Lastly, we observe a significant interaction between ginseng use and gender (*P*-interaction=0.03), with the association evident in men (Ratio: 0.84; 95% CI: 0.72, 0.98), but not women.

DISCUSSION

In a representative sample of US population of adults, we observed use of glucosamine, chondroitin, and fish oil supplements to be associated with reduced inflammation, as measured by hs-CRP. The magnitude of reduction in hs-CRP was 16-22% for these supplements, comparable to what we and others have observed for the association between statin use and CRP (44, 50). Comparison to the effects of aspirin is not possible, because we and others (54-57) have found no clear reduction in CRP with aspirin use, perhaps because aspirin may affect inflammation without affecting CRP (58, 59).

In our study, the percentage of persons reporting glucosamine, chondroitin, and fish oil use is slightly lower than was reported in a recent study of US adults aged 57-85 (60). These differences are largely a reflection of the age of population included, as older adults are more likely to use these supplements. Differences in study years and exclusion of irregular users may also contribute to varying prevalence estimates across studies.

To our knowledge, this is the largest study to investigate the association between use of glucosamine and chondroitin supplements and a marker of inflammation in humans. Our finding of lower hs-CRP levels among users of glucosamine and chondroitin supports laboratory studies which suggest that glucosamine and chondroitin supplementation may reduce inflammation via inhibition of nuclear factor kappa B (NFkB) activation (14, 61-63). NFkB is a transcription factor which lies upstream of many inflammatory processes, including CRP. Laboratory studies have further shown that these compounds also affect factors downstream of NFkB, such as cyclooxygenase activity, as well as pro-inflammatory cytokines interleukin-6 and tumor necrosis factor alpha (14-18, 64-66). Despite this suggestive laboratory evidence, we only know of two small studies which have reported on glucosamine or chondroitin supplement use and inflammation in humans. In a RCT of rheumatoid arthritis patients, Nakamura and colleagues randomized 25 persons to receive glucosamine and 26 to receive placebo for

12 weeks (67). In this study, authors report no effect of glucosamine on CRP; however, persons with rheumatoid arthritis have higher levels of systemic inflammation than the general population and therefore results from this study may not be generalizable to persons without chronic inflammatory conditions (67). A second RCT was conducted in which 36 osteoarthritis patients were given a glucosamine hydrochloride and chondroitin sulfate compound, and seventeen were given placebo. After the three month intervention period, study authors observed a significant decrease in serum prostaglandin E_2 concentrations among those treated with glucosamine (P<0.01) (19). We know of no other human studies to report on the association between these supplements and inflammation.

Our results suggest a biologic mechanism to substantiate epidemiologic observation of an association between glucosamine and chondroitin use and reduced risk of chronic diseases. Based on observational studies within the VITamins And Lifestyle (VITAL) cohort, use of glucosamine and/or chondroitin was associated with reduced risk of colorectal cancer (68) and lung adenocarcinoma (69). Aspirin follows a similar pattern: in combined analyses of RCTs, aspirin use has been shown to reduce risk of both colorectal cancer (7) and death from lung adenocarcinoma (70). The VITamins and Lifestyle study also reported a reduction in total mortality associated with glucosamine and chondroitin use (71); similarly, aspirin use has been associated with reduced total mortality in some observational studies (72) and trials (73). Lastly, in the present study, the associations between glucosamine and chondroitin supplement use and CRP appear to be largely driven by the associations in women, and in the VITamins and Lifestyle study of lung cancer risk, we also observed a greater protective effect of these supplements among women (69). While the biologic mechanism underlying this interaction is unclear, it is feasible that these observed gender differences may reflect differential bioavailability or metabolism by gender (74, 75).

Regular use of fish oil supplements was associated with lower CRP. Fish oil contains long-chain omega-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These omega-3 PUFA are thought to reduce inflammation in several ways, including inhibition of NFkB activation and competitive inhibition of pro-inflammatory omega-6 PUFAs. Omega-3 PUFAs compete with omega-6 PUFAs for the cyclooxygenase-2 enzyme and displace omega-6 stores in cell membranes (24, 76, 77). There have been numerous human trials of omega-3 supplements and CRP or other markers of inflammation, primarily small trials of subjects at high risk of cardiovascular disease (78, 79). Two reviews published in 2006 concluded that the trials were inconsistent and inconclusive (78, 79). More recently, however, two larger RCTs of omega-3 supplementation have reported that the supplements reduced circulating CRP (34, 35) and tumor necrosis factor alpha (35). These studies, plus our current study in a representative US population, provide evidence for the anti-inflammatory effects of long-chain omega-3 PUFAs in humans, and support one of several mechanisms (78, 80, 81) by which long-chain omega-3 PUFA intake may reduce the risk of cardiovascular disease (82), some cancers (68, 83-85), and total mortality (71, 82).

Despite the lack of main effect for ginseng supplementation, we observed significant interaction between gender and ginseng use, with the association with CRP evident among men, but not women. Ginseng has been shown to be associated with reduced NFkB and cyclooxygenase expression in laboratory studies, though this hypothesis has not been widely tested, and to our knowledge, nor has it been studied *in vivo* among humans (28, 29). It is interesting to note that in a cohort study, Yi et al report an inverse association between ginseng use and total mortality, an association which was similarly limited to men (86).

We did not observe significant associations between CRP and any of the following supplements: MSM, pycnogenol-containing supplements, garlic, ginkgo, or saw palmetto. Power may have been limited to

detect associations in less commonly used supplements; it is also possible that these supplements may affect inflammation downstream of CRP or that these supplements may not be associated with inflammation in humans.

This study has allowed us to explore previously unexplored questions in a large, nationally representative population; however, this study is not without limitation. First, glucosamine and chondroitin are often taken together in a single supplement, with about two-thirds of users taking a supplement with both compounds and one-third taking glucosamine only (MSM is also included in some formulations). Thus, the observed associations between glucosamine and chondroitin and CRP in this study are not independent and may be due to the biologic activity of one or both of these supplements. Also, we were unable to assess supplement use on the day of blood collection and did not explore the effect of cumulative dose on inflammation. We were, however, able to ascertain usual frequency of use and were able to limit the definition of use to regular use. While there may be some measurement error in the classification of regular users from non-users, such misclassification would likely be non-differential across the population. The reliability of CRP in short-term studies appears to be good (87), suggesting that one CRP measurement is sufficient to examine the relationship between supplement use and CRP at approximately the same point in time. Even so, we cannot exclude the possibility of measurement error. We were not able to adjust for strength of aspirin dose, as dose was not collected for all study cycles, nor were we able to adjust for the indications of all supplements. However, for those supplements with apparent significant associations (glucosamine, chondroitin, and fish oil), we were able to adjust for the primary indications of use. Further, adjustment for dietary factors was ascertained from 1 or 2-day recall, which may not be representative of true normal diet. As these data were collected in an observational setting, we cannot discount the potential for residual confounding by lifestyle factors. While we might expect specialty supplement users to engage in healthier behaviors, it is important to note that the primary indications for glucosamine, chondroitin and fish oil use are adverse

health conditions (arthritis/joint pain, coronary artery disease). Furthermore, results were robust to multivariate adjustment and the multivariate-adjusted predictors of inflammation in Table 1 correspond well with expectations based on the literature.

In summary, this study adds support to laboratory research and to some human studies which suggest that glucosamine, chondroitin, and fish oil may reduce systemic inflammation. In doing so, this study adds biologic plausibility in support of previous studies which have shown beneficial effects of these supplements on chronic diseases. Given the number of diseases with which inflammation is associated, such as cancer and cardiovascular disease, there is need to find safe and effective ways to reduce inflammation. Research suggests that these three supplements have excellent safety profiles (88-92), supporting their potential role in disease prevention. It is therefore important that the potential anti-inflammatory role of these supplements be further investigated.

Table 1. Distribution of Demographic, Lifestyle, Dietary and Medical Factors and Their Association with C-Reactive Protein (CRP): NHANES 1999-2004

Factor	Raw Number	Weighted %	Unadjusted Geometric Mean CRP (mg/L)		Multivariate Adjusted Geometric Mean (mg/L)[a]	
			Mean	95% CI	Mean	95% CI
Demographic						
Age Group (years)						
25-29	820	9.22	1.31	1.16, 1.48	1.47	1.30, 1.66
30-39	1746	22.16	1.56	1.45, 1.68	1.59	1.49, 1.69
40-49	1952	23.50	1.77	1.62, 1.94	1.72	1.60, 1.85
50-59	1488	18.68	2.10	1.95, 2.27	2.04	1.92, 2.18
60-69	1791	13.51	2.64	2.48, 3.21	2.44	2.28, 2.61
70+	2150	12.93	2.42	2.31, 2.55	2.58	2.42, 2.75
Gender						
Male	4976	48.73	1.57	1.49, 1.65	1.58	1.51, 1.67
Female	4971	51.27	2.28	2.16, 2.41	2.26	2.14, 2.38
Race/Ethnicity						
Non-Hispanic White	5259	74.84	1.86	1.77, 1.95	1.88	1.81, 1.97
Mexican American	2209	6.52	2.04	1.86, 2.23	2.07	1.92, 2.24
Other Hispanic	430	4.97	1.91	1.69, 2.15	1.94	1.73, 2.17
Non-Hispanic Black	1742	9.51	2.37	2.16, 2.60	1.93	1.77, 2.11
Other	307	4.15	1.56	1.30, 1.86	1.81	1.55, 2.12
Education						
Less than High School Graduate	3136	19.38	2.36	2.21, 2.52	2.00	1.88, 2.13
High School Graduate/GED or Equivalent	2357	25.50	2.09	1.99, 2.21	1.95	1.84, 2.06
Some College or AA Degree	2504	28.83	1.92	1.81, 2.03	1.88	1.78, 1.99
College Graduate or Above	1950	26.30	1.46	1.35, 1.58	1.80	1.69, 1.93
Lifestyle						
Smoking History						
Never	4937	49.19	1.81	1.71, 1.91	1.80	1.72, 1.89
Former	2896	27.78	1.97	1.86, 2.09	1.83	1.72, 1.94
Current	2114	23.04	2.03	1.91, 2.15	2.23	2.09, 2.37
Body Mass Index Categories (kg/m^2)						
Underweight (<18.5)	135	1.64	0.75	0.59, 0.96	0.73	0.57, 0.95
Normal Weight (18.5- <25)	2855	31.17	1.08	1.01, 1.14	1.09	1.03, 1.15
Overweight (25-<30)	3711	35.65	1.82	1.72, 1.91	1.86	1.77, 1.96
Obese (30-<35)	1969	19.07	2.94	2.75, 3.13	2.88	2.70, 3.07
Severely Obese (35+)	1277	12.47	5.22	4.83, 5.65	4.85	4.49, 5.23
Leisure Time Physical Activity (MET-minutes/week)	4450	36.34	2.41	2.26, 2.57	2.06	1.94, 2.19
None	2167	24.48	1.91	1.79, 2.05	1.87	1.76, 1.99
Low (>0- <600)	3330	39.18	1.52	1.44, 1.61	1.78	1.70, 1.86
High (600+)						
Dietary						
Vitamin E Supplement Use						
No	8753	86.91	1.91	1.84, 1.99	1.91	1.84, 1.99
Yes	1194	13.09	1.81	1.67, 1.97	1.82	1.68, 1.97
Dietary Fiber Intake (g/day)						
Quintile 1 (≤ 8.4)	1990	19.05	2.25	2.08, 2.43	2.12	1.97, 2.28
Quintile 2 (>8.4-≤12.1)	1989	20.13	2.14	1.98, 2.32	2.01	1.87, 2.16
Quintile 3 (>12.1-≤16.2)	2004	20.67	2.02	1.87, 2.18	1.98	1.76, 2.10
Quintile 4 (>16.2-≤22.1)	1975	19.96	1.82	1.68, 1.97	1.84	1.72, 1.96
Quintile 5 (>22.1)	1989	20.18	1.41	1.30, 1.53	1.61	1.49, 1.74
Dietary Fat Intake (g/day)						
Quintile 1 (≤44)	1990	16.79	1.99	1.83, 2.17	1.78	1.58, 2.01
Quintile 2 (>44-≤60)	1989	18.86	1.94	1.79, 2.11	1.81	1.67, 1.97
Quintile 3 (>60-≤78)	1990	19.65	2.00	1.87, 2.14	1.97	1.87, 2.08
Quintile 4 (>78-≤105)	1989	20.46	1.88	1.75, 2.01	1.93	1.81, 2.05
Quintile 5 (>105)	1989	24.24	1.75	1.60, 1.93	1.98	1.79, 2.18

Total Energy Intake (kcal/day)						
Quintile 1 (≤1336)	1992	16.77	2.28	2.11, 2.46	1.82	1.64, 2.04
Quintile 2 (>1336-≤1708)	1989	18.74	2.08	1.92, 2.26	1.88	1.71, 2.07
Quintile 3 (>1708-≤2113)	1989	19.76	1.92	1.80, 2.05	1.84	1.72, 1.97
Quintile 4 (>2113-≤2693)	1988	21.21	1.89	1.75, 2.04	2.00	1.86, 2.16
Quintile 5 (>2693)	1989	23.51	1.55	1.43, 1.68	1.93	1.75, 2.13
Medication Use						
Aspirin Use[b]						
No	8632	87.58	1.85	1.77, 1.93	1.91	1.83, 1.98
Yes	1315	12.42	2.33	2.15, 2.53	1.85	1.71, 2.00
Non-Aspirin NSAID Use[b]						
No	9605	96.03	1.89	1.81, 1.96	1.90	1.83, 1.98
Yes	342	3.97	2.25	1.85, 2.74	1.81	1.54, 2.12
Statin Use						
No	8760	89.04	1.87	1.79, 1.95	1.93	1.86, 2.01
Yes	1187	10.96	2.16	1.98, 2.36	1.68	1.53, 1.85
Medical History						
Diabetes						
No	8734	91.11	1.82	1.75, 1.90	1.89	1.81, 1.98
Borderline	150	1.22	2.48	1.81, 3.39	1.81	1.39, 2.35
Yes	1063	7.67	3.00	2.67, 3.36	2.02	1.84, 2.21
Heart Disease[c]						
No	9033	92.40	1.84	1.77, 1.92	1.88	1.81, 1.95
Yes	914	7.60	2.74	2.44, 3.08	2.20	2.00, 2.42
Arthritis or Joint Pain Not Due to Injury						
No, Neither	5405	56.71	1.63	1.55, 1.71	1.87	1.78, 1.96
Yes, Either	4542	43.29	2.33	2.22, 2.44	1.95	1.85, 2.05
Memory Loss / Confusion						
No	9114	93.23	1.86	1.79, 1.94	1.89	1.81, 1.97
Yes	833	6.77	2.53	2.26, 2.85	2.11	1.87, 2.37

[a]Adjusted for all factors in Table 1 except arthritis/joint pain not due to injury and memory loss/confusion

[b] Aspirin/ non-aspirin NSAID use defined use of the product every day or nearly every day in the last 30 days among those who report use of pain relievers taken nearly every day for a month or longer

[c]Heart disease defined by report of doctor-diagnosed coronary heart disease, angina, or myocardial infarctio

Table 2. Association of Regular Use[a] of Specialty Supplements with C-Reactive Protein (CRP): NHANES 1999-2004

Supplement	Raw Number[b]	Weighted %	Unadjusted Geometric Mean CRP (mg/L)		Age and Sex-Adjusted		Multivariate Adjusted		Stratified Multivariate Adjusted			
									Men		Women	
			Mean	95% CI	Ratios[c]	95% CI	Ratios[c,d]	95% CI	Ratios[c,d]	95% CI	Ratios[c,d]	95% CI
Glucosamine[e]												
No	9513	95.82	1.90	1.83, 1.98	1	Referent	1	Referent	1	Referent	1	Referent
Yes	361	4.18	1.89	1.60, 2.22	0.83	0.71, 0.98	0.83	0.74, 0.93	0.95	0.83, 1.08	0.73	0.61, 0.88
										P for interaction: 0.05*		
Chondroitin[e]												
No	9651	97.19	1.91	1.83, 1.98	1	Referent	1	Referent	1	Referent	1	Referent
Yes	252	2.81	1.75	1.46, 2.10	0.76	0.62, 0.92	0.78	0.67, 0.92	0.93	0.78, 1.12	0.67	0.53, 0.84
										P for interaction: 0.03*		
Methylsulfonylmethane (MSM)[e]												
No	9807	98.79	1.90	1.83, 1.98	1	Referent	1	Referent	1	Referent	1	Referent
Yes	116	1.21	1.96	1.53, 2.51	0.88	0.68, 1.15	0.87	0.66, 1.15	1.09	0.83, 1.43	0.69	0.46, 1.05
										P for interaction: 0.08*		
Fish Oil[f]												
No	9746	97.84	1.91	1.84, 1.99	1	Referent	1	Referent	1	Referent	1	Referent
Yes	167	2.16	1.49	1.18, 1.89	0.69	0.56, 0.86	0.84	0.71, 1.00	0.86	0.64, 1.16	0.85	0.70, 1.03
										P for interaction: 0.88*		
Garlic												
No	9595	96.78	1.90	1.83, 1.98	1	Referent	1	Referent	1	Referent	1	Referent
Yes	296	3.22	1.90	1.58, 2.28	0.94	0.78, 1.13	0.97	0.83, 1.13	0.98	0.81, 1.20	0.96	0.79, 1.17
										P for interaction: 1.00*		
Ginseng												
No	9478	95.57	1.92	1.85, 2.00	1	Referent	1	Referent	1	Referent	1	Referent
Yes	370	4.43	1.58	1.36, 1.84	0.85	0.74- 0.99	0.92	0.81, 1.04	0.84	0.72, 0.98	1.06	0.87, 1.28
										P for interaction: 0.03*		
Pycnogenol (Grapeseed/ Pine Bark)												
No	9760	98.10	1.91	1.83, 1.99	1	Referent	1	Referent	1	Referent	1	Referent
Yes	142	1.90	1.66	1.33, 2.06	0.84	0.67, 1.06	0.88	0.73, 1.06	0.88	0.71, 1.09	0.90	0.69, 1.19
										P for interaction: 0.60*		
Ginkgo[g]												
No	9571	96.42	1.92	1.84, 2.00	1	Referent	1	Referent	1	Referent	1	Referent
Yes	297	3.58	1.58	1.32, 1.89	0.79	0.67, 0.93	0.91	0.80, 1.03	0.88	0.76, 1.02	0.95	0.75, 1.19
										P for interaction: 0.40*		
Saw Palmetto[h]												
No	4803	97.09	1.59	1.51, 1.67	1	Referent	1	Referent	1	Referent		
Yes	137	2.91	1.27	1.03, 1.58	0.74	0.59, 0.93	0.85	0.69, 1.06	0.85	0.69, 1.06		

[*] Two -sided P for interaction tested at alpha=0.05 level
[a] Regular use defined as use in the past 30 days with reported frequency of use of 20+ days/month
[b] Does not total to 9947 for each supplement, as persons were excluded from supplement-specific analyses if missing information on frequency of use or if reported usual use on <20 days per month
[c] Ratio of CRP among those who report regular use of a given supplement as compared to those who report no use/irregular use
[d] Adjusted for age, gender, race/ethnicity, education, smoking history, body mass index, physical activity, vitamin E supplement use, dietary fiber intake, dietary fat intake, total energy intake, aspirin use, non-aspirin NSAID use, statin use, diabetes, and coronary heart disease
[e] Multivariate model additionally adjusted for arthritis and/or joint pain not caused by injury
[f] Multivariate model additionally adjusted for arthritis and/or joint pain not caused by injury, as well as memory loss/confusion
[g] Multivariate model additionally adjusted for memory loss/confusion
[h] Analyses limited to men

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CONCLUSION

In this dissertation project, we aimed to better understand the associations between glucosamine, chondroitin, and fish oil supplement use and CRC. To achieve these aims, we conducted an in-depth analysis of the associations between use of these specialty supplements and CRC and examined the biologic associations by which these associations may reduce risk of CRC.

With extended follow-up in our prospective cohort study, we observed high use (defined as use on 4+ days/week for 3+ years) of glucosamine+chondroitin to be marginally associated with reduced risk of CRC. The association between glucosamine+chondroitin and CRC varied by body mass index, with a significant inverse association observed among the overweight and obese, but not among persons of lower body mass index. Since we hypothesized that the posited anti-inflammatory supplements, glucosamine and chondroitin, may act to reduce risk of CRC most among persons of higher inflammation, such as those with body mass index, this finding adds further biologic plausibility to the observed association. The association between glucosamine and CRC was also marginally significant, though this association got weaker when exposure was defined in terms of glucosamine alone, suggesting that the observed association may be driven by chondroitin or by the combination of glucosamine+chondroitin.

Use of fish oil supplements was also observed to be marginally associated with CRC, with high users having about half the risk of CRC as non-users. This association varied by gender and subsite, with associations driven by men (rather than women) and by cancers of the colon (rather than cancers of the rectum). While total EPA+DHA and dark fish consumption were not associated with CRC overall, these associations were modified by underlying genetic risk, with significant inverse associations observed among persons of low and moderate genetic risk and positive associations observed among persons of high genetic risk.

In analyses of both glucosamine+chondroitin and fish oil supplements, we observed that the overall associations were weaker than was observed in the initial exploratory analysis. If a genuine association exists and the etiologically relevant time frame extends into follow-up, then changes in exposure status would act to increase measurement error, thereby attenuating effects. Understanding the reason for this attenuation may help us understand the etiologically relevant time frame by which these supplements may act to influence carcinogenesis, though further study will be needed in order to fully address this question.

We observed use of glucosamine and chondroitin supplements to be associated with reduced oxidative stress, as measured by PGF2α. However, use these supplements was not associated with 8-isoprostane, DNA damage, or DNA repair capacity, and it remains unclear why associations were not consistent across biomarkers. However, it seems plausible that factors associated with oxidative stress may not be associated with DNA damage, as any one factor, such as oxidative stress, may not make a large enough relative contribution to DNA damage to see effect. It should also be noted that in analyses of oxidative stress and CRC, the association between glucosamine and CRC weakened when the definition of use was limited to glucosamine alone, suggesting that perhaps chondroitin or the combination of glucosamine+chondroitin is driving observed associations. Use of fish oil supplements was not associated with measures of oxidative stress or DNA damage. Glucosamine, chondroitin, and fish oil supplements were all observed to be associated with reduced systemic inflammation, as measured by hsCRP. Given the overlapping nature of inflammation and oxidative stress, it remains difficult to parse apart whether glucosamine and chondroitin act primarily to reduce inflammation, oxidative stress, or both.

Results of this study suggest biologic mechanisms by which glucosamine, chondroitin, and fish oil supplements may reduce the risk of CRC. One of the major strengths of this study is that our

observations are not limited to one study population. However, further research in additional study populations is needed, as consistent observation across populations will add further support to these findings. As a next step, we plan to assess whether use of these supplements is associated with various markers of inflammation with the VITAL biomarker study, with biomarkers of inflammation including IL-1β, IL-6, IL-8, TNFα, CRP, TNFRI, TNFRII, and PGE-M. This study will offer the opportunity to replicate our finding from NHANES within a different population using additional biomarkers. Furthermore, a small pilot trial is underway to better understand the biologic effects of glucosamine and chondroitin supplementation. This trial will obviate typical concerns of observational studies, including concerns related to residual confounding by factors associated with health behaviors. Taken together, the body of work presented in this dissertation in conjunction with these additional biomarker studies may lay the groundwork for future study of these supplements as potential chemopreventive agents.

If glucosamine, chondroitin, and fish oil act to reduce inflammation and/or oxidative stress, it is possible that these supplements may affect other outcomes, beyond CRC, for which these biologic mechanisms play an important role. We have already observed that glucosamine and chondroitin are associated with reduced risk of lung cancer (1, 2) and total mortality (3, 4), while fish oil has been associated with reduced risk of breast cancer (5) and heart disease (6). Future observation of an association with other related outcomes or observation in other study populations may generate further support for the effects of these supplements.

Lastly, results of our analyses also suggest certain subgroups may benefit more or less from use of these supplements. The observed differences across subgroups (such as gender, BMI, genetic risk) is not only important to understanding the chemopreventive potential of these supplements, but may also shed light on the mechanisms by which these supplements may affect cancer risk.

These results from this project are important as they build the foundation of research involving these supplements in disease prevention. Beyond the posited effect of glucosamine, chondroitin, and fish on inflammation and/or oxidative stress, and CRC, these supplements have been suggested to have excellent safety profiles (7-11), further supporting their potential role in this setting.

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