ABSTRACT

Title: **RELATIONSHIP BETWEEN FISH INTAKE, OMEGA-3 FATTY ACIDS, MERCURY, AND RISK MARKERS OF CORONARY HEART DISEASE (NHANES 1999-2002)**

Kimberly Michele Smith, Masters of Science in Nutrition, and 2006

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Background: Fish consumption is inversely associated with coronary heart disease (CHD), possibly due to the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Dietary mercury from fish has been hypothesized to increase the risk of CHD.

Objective and Design: Test the relationships between fish FFQ/30 days and 24-hour DHA+EPA intakes and CHD risk markers (high density lipoprotein (HDL), low density lipoprotein, total cholesterol, triacylglycerol, and C-reactive protein (CRP)) in F 16-49 y (NHANES 99-02), and if mercury attenuated any cardio-protective benefits.

Results: Fish FFQ was negatively associated with CRP (95% CI: -0.19 to -0.02, $p=0.015$) and positively associated with HDL (95% CI: 0.31 to 2.5, $p=0.014$), and was not significantly attenuated after adjustment for multiple other risk factors. Addition of mercury resulted in non-significant relationships between fish FFQ and HDL (95% CI: -0.60 to 1.6, $p<0.05$). DHA+EPA and other nutrients in fish may be adequate to offset the hypothesized CHD risks of mercury.

RELATIONSHIP BETWEEN FISH INTAKE, OMEGA-3 FATTY ACIDS, MERCURY, AND RISK MARKERS OF CORONARY HEART DISEASE (NHANES 1999-2002)

By

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TABLE OF CONTENTS

List of Tables

List of Abbreviations

CHD	Coronary Heart disease
DHA	C22:6n-3 docosahexaenoic acid
EPA	C20:5n-3 eicosapentaenoic acid
NHANES	National Health and Nutrition Examination Survey (1999-2002)
TBHg	Total blood mercury
CRP	C-reactive protein
TC	Total cholesterol
HDL	High density lipoprotein cholesterol
LDL	Low density lipoprotein cholesterol
TG	Triacylglycerol
AHA	American Heart Association
NCHS	National Centers for Health Statistics
Hg	Mercury
FFQ	Food frequency questionnaire
JTG	Joint task group
FDA	Food and Drug Administration
EPA	Environmental Protection Agency
MI	Myocardial infarction
RDA	Recommended dietary allowance
CVD	Cardiovascular disease
PUFA	Polyunsaturated fatty acids
BMI	Body mass index
AT	Alpha tocopherol
ROS	Reactive oxygen species

Chapter 1: Introduction and Literature Review

Introduction

The major aim of this study was to use the most recent National Health and Nutrition Examination Survey (NHANES 1999-2002) data (1) to investigate the relationship between frequency of fish consumption and the marine-based omega-3 fatty acid [(eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] intake, and risk markers of coronary heart disease (CHD) and the effects of total blood mercury (TBHg) on these relationships. The study was limited to female participants aged 16-49 years for whom data on TBHg were available. The tested hypotheses were as follows: (1) 30-day frequency of fish intake and 24-hour DHA+EPA intake are negatively associated with serum concentrations of total cholesterol (TC), LDL cholesterol (LDL), triacylglycerol (TG), and C-reactive protein (CRP), and positively associated with high density lipoprotein cholesterol (HDL) concentrations and, (2) TBHg attenuates the cardio-protective benefits of DHA+EPA as assessed by TC, LDL, TG, HDL and CRP concentrations, controlling for multiple known confounders.

To date, seven studies have addressed the combined effects of omega-3 fatty acids (from food or dietary supplement sources) or fish intake and marine derived mercury (Hg) on the risk of CHD. Five studies used male participants only; one used female participants only; and one evaluated both men and women. Of the seven studies, three used subjects from the high fish consuming populations in Finland, a fourth study was conducted in men from eight European countries, a fifth study looked at male health professionals within the US, the sixth investigated both men and women from Sweden, and the seventh was conducted in Swedish women only. Four of the seven studies reported that Hg might attenuate the cardio-protective effects derived

from fish consumption (2-5). Two studies failed to demonstrate a significant association between Hg and CHD (6, 7) and one study reported a negative association between measured Hg concentrations and CHD (8). These studies are reviewed in detail further in the text.

Six of the seven previous reports that evaluated the combined effects of fish (or DHA+EPA) and Hg on CHD were performed only in male subjects, while one study also included women. The method of assessing Hg exposure (including measurements in hair, serum, blood and toenail) varied according to study. The hypothesis that consuming fish with high Hg concentrations may diminish the cardio-protective effects of fish remains uncertain particularly with respect to women. Our study extends the work of previous researchers and contributes to the ongoing scientific and public health policy debate on the benefits and risks of fish consumption, particularly in women.

Evaluation of Existing Knowledge and Availability of Data

The most recent NHANES data (1999-2002) reported by the National Centers for Health Statistics (NCHS) included measurements of blood, hair, and urine Hg (total and inorganic) for women of childbearing age (16-49 years) to assess Hg exposure from fish. Blood and hair measures were also included for children aged 1-5 years. The NHANES (1999-2002) included a 24-hour food intake history from which DHA+EPA intake was derived, and a 30-day fish food frequency questionnaire (FFQ) for 31 varieties of fish and shellfish. Shellfish were excluded from the analysis because few shellfish contain Hg (9).

In the current study using the NHANES (1999-2002) data, there were five dependent variables of interest related to CHD risk, including 4 lipid biomarkers (LDL, HDL, TC, TG) and the inflammatory marker, CRP. In addition, the NHANES (1999-2002) data set includes a variety of independent variables known to affect CHD risk including dietary nutrient intake,

physical activity, diabetic status, smoking status, use of prescription medications, and age. The analysis target sample was limited to women aged 16-49 years for which TBHg was measured. The risk markers for CHD and other variables known to affect CHD risk are reviewed in detail below.

Risk Markers of CHD

Abnormal blood lipid levels contribute significantly to the risk of CHD, and this is compounded by other risk factors (10). The dependent variables serving as risk markers in the current study were serum LDL, HDL, TC, TG, and CRP concentrations. The use of both lipid and inflammation biomarkers to assess risk in postmenopausal women is supported by recent research. Ridker and colleagues demonstrated that models in apparently healthy postmenopausal women, which incorporated markers of inflammation as well as lipids, were significantly better at predicting CHD risk in apparently healthy postmenopausal women than models based on lipid levels alone ($p<0.001$) (11).

C-reactive protein

CRP is considered one of the best measures of acute phase response to an infection, disease, or other cause of tissue damage and inflammation and is well established as a predictor of cardiovascular disease (11). Multiple studies have demonstrated that elevated inflammatory markers, including CRP, indicate an increased risk of CHD (12). CRP can be used to measure the body's response to chronic conditions including arthritis and chronic smoke inhalation (1). Since many markers of inflammation may not be tissue specific, there is error involved in using some markers as independent predictors of a particular disease.

CRP is affected by numerous lifestyle variables. In general, individuals with elevated CRP tend to smoke, have high blood pressure, are overweight, and fail to exercise (13). Elevated concentrations of CRP in blood suggest an acute infection or inflammation. The

clinical cutoff points established for CRP are “low” (<1.0 mg/L), “moderate” (1.0 to 2.9 mg/L) and “high” (≥ 3 mg/L) (14).

CRP was shown to be a valuable predictor of CHD risk in women. Female participants in the Nurses Health Study who suffered from CHD during 8 years of follow-up had significantly higher CRP concentrations than healthy controls (3.1 mg/L vs 2.2 mg/L, $p<0.001$) even after adjusting for multiple other CHD risk factors (12). The Women’s Health Study enrolled 27,939 apparently healthy post-menopausal women aged ≥ 45 years between 1992 and 1995 who were then followed up for 9 years or until the first cardiovascular event. Compared to women with baseline CRP concentrations of <1 mg/L, the crude relative risk for post menopausal women with CRP concentrations of 1.0 to 2.9 mg/L was 1.7 (95% CI, 1.4 to 2.2) whereas the relative risk (RR) was greatest for those with CRP concentrations ≥3 mg/L (3.0, 95% CI, 2.4 to 3.7) (p for trend across groups was <0.001) (15). The predictive value of CRP demonstrated similar effects in male subjects.

A newer method of measuring CRP is available and is called high sensitivity CRP analysis [(hs) CRP]. Both CRP and (hs) CRP methods measure the same molecule in the blood. The (hs) CRP method has been used to determine risk of CVD for seemingly healthy people and ranges from 0.5 to 10 mg/L. The CRP test is ordered for patients at risk for bacterial or viral infection (such as following surgery) or patients with chronic inflammatory diseases and ranges from 10 to 1000 mg/L. The NHANES (1999-2002) documentation does not specify that the (hs) CRP test was used; however, the levels reported in the female participants aged 16-49 years are consistent with a (hs) CRP test rather than CRP (16).

Other markers of risk proposed for use in screening include homocysteine, fibrinogen, fibrinolytic capacity, and levels of apolipoprotein A-1, apolipoprotein B-100, and Lp(a)

lipoprotein. These biomarkers have limited clinical use, however, because there are inadequate standardization of assay conditions, prospective data are inconsistent or non-existent, and these markers, when incorporated into prediction models, did no better than conventional lipid screening in predicting risk (17).

Total cholesterol

In a prospective cohort study (EUROASPIRE), men and women with established CHD were followed from baseline for five years. Patients who died of CHD related causes had significantly higher total cholesterol at baseline than patients who survived (6.61 mmol/L vs 6.07 mm/L, $p=0.002$). In addition, patients who died had lower HDL concentrations at baseline than survivors (1.20 mmol/L vs 1.23 mm/L), however the difference was not significant (18). Based upon the results of the Framingham Study, researchers reported that screening for TC alone in women aged 50 years and older may not be adequate for identifying individuals at risk for CHD (19).

HDL cholesterol

Low concentrations of HDL are often associated with a non-healthy lifestyle such as, smoking, lack of regular exercise, and being over weight. A strong inverse relationship exists between plasma HDL concentrations and CHD risk in both sexes (19). Rates of MI were significantly greater for female Framingham participants (aged 50 to 79 years) in the lowest quartile of HDL concentration (23 to 46 mg/dL) compared to those in the highest HDL quartile (67 to 139 mg/dL) ($p<0.05$). After adjusting for age and other risk factors, participants in the lowest HDL quartile experienced nearly six times the number of coronary events compared to those in the highest quartile of HDL concentrations ($p<0.001$) (19).

LDL cholesterol and triacylglycerol

Other blood lipids such as LDL and triacylglycerol (TG) are valid predictors of CHD risk. However, triacylglycerol was only measured in a subset of the population in NHANES. Since LDL calculation is based on TG, these variables were limited to a subset of the population.

Factors that may affect these CHD risk markers along with variables that were evaluated as confounders in the current research are described below.

Comment [NRS1]: Something like that. --Ok

Omega 3 fatty acids

Previous reports on the relationship between marine derived omega-3 fatty acids and the risk of CHD have varied according to age, gender, and disease state of the subjects, with the majority of the studies conducted in men. Observational studies have shown that the inverse association of fish or marine omega-3 fatty acid intake with CHD is stronger in high risk versus low risk populations (20). The NHANES sample used in this study included women aged 16-49 years in whom the CHD risk varied greatly. Because age is a risk factor for CHD, the subjects in this study were stratified by decade (ie., 20-29, 30-39 and 40-49 years), with subjects aged 16-19 years used as the reference group.

Established relationships between omega-3 fatty acids, fish and CHD

In 2000 a Joint Task Group (JTG) consisting of the Consumer Healthcare Products Association, Council for Responsible Nutrition and National Fisheries Institute conducted a systematic review of publications related to omega-3 fatty acids and CHD, in support of a proposed qualified health claim for omega-3 fatty acids. The JTG found 16 observational studies and rated 9 as “good to moderate”. These nine studies all reported a negative association between omega-3 fatty acids and CHD. Studies that did not demonstrate protective effect of omega-3 fatty acids on CHD had limitations such as short duration time or lack of adequate follow up. Prospective cohort studies using initially healthy populations generally require a

minimum of 10 years of follow-up since CHD is a slowly progressive disease. One study evaluated women, all of whom were older adults (all born before 1907).

Dietary supplements of omega-3 fatty acids vary widely according to dose and the ratio of EPA to DHA. A 12-week parallel double-blinded supplementation study that provided 1.5 g/day omega-3 fatty acids (EPA/DHA ratio of 0.67) resulted in a non-significant association with markers of inflammation (CRP) in apparently healthy male and female volunteers aged 20 to 55 years (21). A recent study evaluated the effects of 2.8 g/day DHA supplementation (alone) on cardiovascular disease risk factors in post menopausal women (aged 45-79 years) using a double blind, placebo controlled cross over design. The subjects recorded their own dietary intake during the two 28-day study periods (placebo or DHA supplement). Data on physical activity were not collected. The authors found that DHA supplementation had no significant relationship with blood levels of CRP, but raised HDL concentrations by 8% ($p=0.017$) (22). When healthy post- menopausal women were supplemented with 14g/day or 14 g/day safflower oil, those consuming fish oil had significant decreases in CRP, TG and the ratio of TG to HDL (23). Supplements in the range of <1g/day to 4g/day taken by different samples of subjects have produced varying results on biomarkers of inflammation. Intervention trials have not found a significant association between omega-3 fatty acids (both marine and plant based) and CRP. Male and female type 2 diabetic subjects with an average aged of 61.2 years who were supplemented with 4 g/day DHA+EPA did not have significant changes in CRP concentrations (24). Whereas observational studies as described above have seen significant associations between these fatty acids and CRP (25). Only 1.6% of participants in the Nurses Health Study (women aged 30-55 years) reported taking omega-3 fatty acid supplements, suggesting that this type of supplement is not often used by middle aged women (26).

Fish consumption has been shown to be cardio-protective in women. Among female participants in the Nurses Health Study (aged 30-55 years), fish consumption was inversely associated with the incidence of CHD (p=0.003) (26). Compared to women who seldom ate fish (<1 serving per month) the relative risks of CHD (adjusted for age, smoking and other known CHD risk factors) were 0.70 (95% CI: 0.48 to 1.03) for fish consumption 1 to 3 times per month, 0.60 (95% CI: 0.42 to 0.85) for once per week, 0.64 (95% CI: 0.42 to 0.99) for 2 to 4 times per week and 0.36 (95%: CI 0.20 to 0.66) for five or more times per week (p for trend= 0.002) (27). Despite the evidence that fish consumption is beneficial for cardiovascular health and tends to increase with age (28) fish intake remains low in the US (1).

A recent meta-analysis quantified CHD risk as a function of fish consumption (number of servings/week) and took into account any risks derived from marine based methyl Hg exposure. The authors concluded that consuming >1 serving of fish per month is associated with a 17% reduction in CHD mortality risk, with an additional 3.9% risk reduction for each additional serving of fish per week (29).

Mechanism of omega-3 fatty acids on CHD

Omega-3 fatty acids protect against CHD by a variety of physiological mechanisms, which include lowering plasma TG concentrations, inhibiting plaque formation, decreasing platelet aggregation and altering arrhythmogenesis (30). These effects have occurred in humans and animals with both food and dietary supplement sources of omega-3 fatty acids.

Methyl Mercury

Total Hg (often measured in hair and blood) is composed of inorganic and organic Hg. There are multiple sources of exposure to inorganic Hg including dental amalgams, “folk” or patent medicine, cosmetic preparations, Hg spills in homes or schools, select plant based foods,

trace amounts in eggs, organ meats, and kidney, and occupational exposure (31). Organic Hg (methyl Hg) is present in fish due to its uptake from environmental sources. It occurs in soil, rocks, streams, lakes and oceans as a result of burning fossil fuels. Other sources of organic Hg are industrial wastes such as thermometers, batteries, electrical switches, Hg based paint, pesticides, and fungicides, and byproducts of the mining and pulp and paper processing industries (32). In humans, the sole source of exposure to organic Hg is the consumption of fish and sea mammals (33). Organic Hg found in fish and shellfish has repeatedly been confirmed as being methyl Hg (31). Most fish have trace amounts of Hg because of the metals' tight protein binding properties. Mercury levels in fish vary depending on the aquatic environment (e.g., proximity to industrial mercury sources) and place in the food chain. Large long-living predatory fish have been shown to contain the greatest amounts of methyl Hg (9). In this study, references to dietary Hg are organic methyl Hg (Hg) unless indicated otherwise.

Risk of Mercury Intake

The risk associated with eating fish contaminated with Hg varies among the population. The Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) jointly released a revised consumer advisory on Hg toxicity in fish aimed at the most sensitive groups, including pregnant women, nursing mothers, women who may become pregnant, and young children (34). Mercury has adverse neurological effects on the developing fetus and can retard psychomotor development in young children (35). Research has linked Hg ingestion from fish to increased risk of CHD (2-5, 7). The cardiovascular effects of Hg from fish are not included in the government advisory statements.

Mechanism of action

NHANES (1999-2002) provided total blood Hg for a subset of women aged 16-49 years. Ninety percent of Hg is located in red blood cells and a small percentage is metabolized into Hg ions (32). Erythrocytes and plasma contain even distributions of metallic and inorganic Hg whereas organic Hg is greater in erythrocytes than in plasma (35, 36). All exposure to Hg (inorganic and organic) is reflected in blood. After Hg is ingested it can easily pass through cellular membranes, resulting in neurotoxic effects (35). Once absorbed, inorganic Hg is excreted in the urine (32). NHANES (1999-2000) provided data on urinary Hg concentrations, which reflect exposure to inorganic Hg from environmental sources rather than from the dietary intake of organic Hg from marine sources. Therefore, urinary Hg data were not included in the current study. Approximately 90% of absorbed organic Hg is excreted in the feces, but fecal biomarkers were not collected by NHANES. Once absorbed, the average half-life of Hg in humans is 70 days, but it can vary among individuals from 35 to 189 days (35, 36). Thus, the amount of Hg measured in total blood in NHANES is representative of the fish reported consumed in the NHANES 30-day fish FFQ.

Established relationship of mercury and fish consumption

The half life of Hg in blood, which is >30 days, allows the use of total blood Hg levels measured in NHANES to represent Hg derived from fish, as reported in the 30-day FFQ and the 24-hour dietary recall. Studies to date have demonstrated significant positive relationships between fish consumption and Hg levels. Three studies have been published using the NHANES (1999-2000), all of which found positive associations between Hg (in hair and blood) and fish intake.

The relationship between hair Hg concentrations, blood Hg levels, socio-demographic variables, and 30-day fish FFQ responses was investigated using the NHANES (1999-2000)

(37). The subjects were 1,726 females aged 16-49 years, including 292 pregnant women. Fish FFQ responses were analyzed according to type (fish or shellfish) and frequency (0 times, 1-2 times, or >3 times in 30 days). The mean hair Hg concentration was 0.47 ug/g (95% CI: 0.35 to 0.58). When analyzed by age, mean hair Hg increased with each decade. For example, compared to women ages 16-19, women 30-39 and 40-49 years of age had significantly greater hair Hg levels ($p<0.05$).

Prior to the release of the 1999-2000 NHANES hair Hg data, the NHANES only included data on blood Hg levels. Schober et al, (2003) published the initial report on total blood Hg data (NHANES 1999-2000) (38). The geometric mean concentration of total blood Hg for the 1,709 women was 1.02 ug/L (95% CI, 0.85-1.20 ug/L). Women who ate 3 or more servings of fish per month had a four-fold greater geometric mean Hg level compared to non fish consuming women (1.94 ug/L vs 0.51 ug/L; $p<0.001$).

The NHANES did not provide organic Hg measurements but only total blood Hg concentrations. However, MaHaffey et al (2004) described the association between total blood Hg levels and the calculated organic Hg intake derived from the methyl Hg in fish and shellfish (1999-2000 NHANES) (31). Fish were the major source of organic Hg. Fish consumption was derived from the NHANES 30-day fish FFQ. The Hg content of fish and shellfish were derived from the National Marine Fisheries Service database. The authors multiplied fish intake per eating occasion (39) by the 30-day fish FFQ to determine average monthly fish consumption in grams. Average monthly fish intake was combined with the Hg content of fish to determine estimated monthly organic Hg intake. These values were compared to the total blood Hg NHANES data. Blood organic/methyl Hg was greatest for participants who classified themselves as “other racial/ethnic category” which included Asians, Native Americans, and

Pacific Islanders (geometric mean=1.06, 95% CI: 0.18-1.34). Blood organic/methyl Hg concentrations increased by approximately 0.2 ug/L per decade of age. This increase was consistent with findings from the other blood and hair Hg analyses previously conducted. Women who reported at least 9 occasions of fish or shellfish consumption within the past 30-days had total blood Hg concentrations almost seven times higher than non-consumers (2.49 ug/L compared to 0.39 ug/L). The calculated blood organic Hg was greatest for those who reported consuming fish > 9 times per week (2.70 ug/L, 95%CI: 1.51-3.89); however, this group also comprised the smallest sample (n=63). The adjusted multiple correlation coefficient (R) for the association between quantity of fish and shellfish eaten per kg of bodyweight and blood Hg was 0.54 for total blood Hg and 0.55 for calculated organic blood Hg. The authors separated consumption by type of fish. Tuna (one of the two most commonly consumed fish), and "other fish" were both significant predictors of blood Hg ($p<0.05$). Overall, shellfish consumption (log shellfish) had minimal to no effect on total blood Hg and organic blood Hg. However, each gram of shellfish and fish consumed (per kg bodyweight of the subject) resulted in a 0.2 to 0.5 ug/L increase in blood Hg concentrations.

Several prospective studies reported significant associations between fish consumption and Hg biomarkers in men (2-5, 40). One study was conducted with 1,833 middle-aged men in the Finland Health Registry who were followed prospectively for 7 years. The men reported an average fish intake of 46.5 g/day and had hair Hg concentrations of 2 mg/kg. Men in the highest tertile of hair Hg content (>2.0 µg/g) had a 2.0-fold greater age- and CHD-adjusted risk of acute MI (95% CI: 1.2 to 3.1; P=0.005) and a 2.9-fold greater adjusted risk of cardiovascular death (95% CI, 1.2 to 6.6; P=0.014) compared with those with a lower hair Hg content (<2.0 ug/g) (3).

A study conducted in Finnish men aged 42-60 years without prior CHD or stroke found that fish intake in men with the highest tertile of hair Hg was more than twice that of men in the lowest hair Hg tertile (65 vs 30 g/day; $p<0.001$). High hair Hg was strongly associated with fish consumption ($r=0.27$; $p<0.001$) and serum DHA + EPA concentrations ($r=0.25$; $p<0.001$) (2).

Guallar et al (5) evaluated the relationship of Hg measured in toenails and DHA measured in adipose tissue on the risk of having a first MI. Concentrations of DHA in adipose tissue were strongly correlated with toenail Hg ($p<0.0001$). Similarly, a nested case control design in male health professionals aged 40-75 years found a positive relationship between fish consumption and toenail Hg concentrations ($r=0.42$, $p<0.001$) (40). In a nested case control study conducted on Swedish men and women aged 30-60 years, erythrocyte Hg was significantly greater in subjects who reported $\geq$ 1 fish meal per week compared with those reporting <1 fish meal per week, irrespective of the type of fish (lean or fatty) consumed ($p<0.001$) (7).

Effects of mercury on risk of CHD

Seven studies have investigated the combined effects of omega-3 fatty acids (from food or dietary supplements) or fish intake and marine derived Hg on the risk of CHD. Five studies used male participants only; one used only females; and one evaluated both men and women. Of the seven, three were conducted on the high fish consuming populations in Finland, a fourth study was conducted in men from eight European countries, a fifth study looked at male health professionals within the US, the sixth investigated both men and women from Sweden, and the seventh was conducted in Swedish women. Four of the seven studies reported that Hg might attenuate the cardio-protective effects demonstrated by fish consumption (2-5). Two studies

failed to demonstrate a significant association between Hg and CHD (6, 7) and one study reported a negative association between Hg concentrations and CHD (8).

One of the first studies was conducted with 1,833 middle-aged men selected from the Finland Health Registry. Over the 7-year observation period, average fish intake was 46.5 g/day and hair Hg was 2 mg/kg. Men with hair Hg in the highest Hg tertile (2ppm or higher) had twice the risk of an acute myocardial infarction than the remainder of the study sample (RR 1.7, p=0.038) (3).

A follow up study of the Finnish cohort of 1,871 middle-aged men conducted in 2000 prospectively measured marine derived omega-3 fatty acids. Participants in the upper quintile of omega-3 fatty acid intake and having low hair Hg concentrations (<2 ppm) demonstrated a 67% reduction in MI risk (p=0.016). Conversely, participants in the highest quintiles of omega-3 fatty acids and high hair Hg demonstrated a 24% reduction in risk (4). Men in the two lowest tertiles of hair Hg who were also in the highest quintile of serum DHA + EPA had a 67% reduced risk of acute coronary events compared to men in the highest third of hair Hg and lowest quintile of serum DHA+EPA (95% CI: 19% to 87%; p=0.016). The authors concluded that high Hg in fish may attenuate the cardio-protective effects of omega-3 fatty acids in middle aged men (4).

A third study, conducted in Finnish men aged 42-60 years without prior CHD or stroke (n=1,871), investigated the effects of elevated hair Hg content on the risk of acute coronary events and cardiovascular all-cause mortality. Subjects with “high” hair Hg content (>2.03 ug/g) had a 1.60 fold greater adjusted risk of an acute coronary event (95% CI: 1.24 to 2.06) and a 1.68 fold greater risk of CVD (95% CI: 1.15 to 2.46) than the lower two thirds. The authors concluded that hair Hg levels greater than 2.03 ug/g may be a risk factor for acute coronary

events. It also may increase the risk of CVD, CHD and all cause mortality in middle aged eastern Finnish men, as well as attenuate the protective effects of fish (as assessed by serum DHA levels) on cardiovascular health (2).

Guallar et al. (5) evaluated the relationship of Hg measured in toenails and DHA measured in adipose tissue on risk of first myocardial infarction (MI). After adjustment for age, location and DHA, MI patients had higher toenail Hg than the healthy controls [odds ratio; 1.10 (95% CI: 1.03-1.18)]. The researchers concluded that high toenail Hg diminished the cardio protective effect of fish intake.

A nested case control design in male health professionals aged 40-75 years failed to demonstrate a significant relationship between Hg and CHD. The researchers found a positive relationship between fish consumption and toenail Hg ($r=0.42$, $p<0.001$) levels but no significant increase in risk of CHD with higher toenail Hg concentrations even after adjusting for age, smoking and other risk factors (40).

Conversely, a population based case control study in both men and women, aged 30-60 years, found a significant effect of blood Hg on risk of MI. In a multivariate model, the risk of MI was lower for subjects with both high erythrocyte Hg and high plasma DHA+EPA (OR= 0.16, 95% CI: 0.04 to 0.65) than for plasma DHA+EPA alone (7). This association may be more indicative of the benefits of fish consumption rather than a protective effect of blood Hg.

Finally, a study conducted in 1,462 Swedish women aged 38-60 years at baseline found a protective effect of serum Hg. The authors followed the women for 24 years. After controlling for age and education, the correlation between MI and serum Hg was less than zero, indicating a protective effect of Hg. The authors did not control for omega-3 fatty acids or fish consumption (8).

In summary, the majority of the studies to date that evaluated the combined effects of fish (or DHA+EPA) and Hg on CHD were case control studies using male subjects. One of the six studies also included women. The majority of the studies in men demonstrated a positive association between Hg and CHD; however, additional research is needed on the benefits from fish as well as fish variety (e.g. lean versus fatty fish).

Variables other than omega-3 fatty acids and methyl mercury that may affect risk markers of CVD

Age and BMI

It is well understood that the risk of CHD increases with age as well as with an increase in non-lean body weight. Body mass index (BMI) is often used as a measure of weight for height and is calculated as weight (kg) divided by height squared (m^2). BMI is not the most accurate measure of healthy body weight because it does not account for the difference in lean versus non-lean tissue. It can be inaccurate for the young, the elderly, and athletes who have high muscle mass, as muscle tissue is denser than fat tissue.

Diabetes, arthritis, stress, trauma and pregnancy

Obesity, insulin resistance, diabetes, hypertension, and low HDL concentrations are all associated with increased production of inflammatory markers including CRP (12). Diabetes is a disease associated with impaired glucose metabolism and insulin resistance, and diabetics are at a high risk for atherosclerosis and cardiovascular related diseases (41). Other conditions associated with inflammation and inflammatory biomarkers include arthritis, burns, sepsis, surgery, pregnancy and any type of trauma (42).

Medical history of coronary heart disease

Dyslipidemia is associated with an increased risk of CHD and is characterized by elevated levels of LDL (≥160 mg/dL), TG (≥200 mg/dL) and TC (≥240 mg/dL), and a low level of HDL (≤40 mg/dL) (16).

Antioxidant Intake

Epidemiological studies investigating the role of antioxidants in reducing the risk of CHD have produced inconsistent results. Clinical studies conducted in 1999 to 2002 typically focused on dietary intakes of vitamins E, C, and B-carotene in post-MI or other high-risk patients. Five studies which evaluated vitamin E supplementation, in doses ranging from 300 mg of synthetic to 400 IU of natural vitamin E, had no effect in reducing the risk of MI, CHD, death or stroke for high risk (post MI and diabetic) adults. Similarly, no effects were seen in studies using B-carotene or mixtures of antioxidants (43).

Because diabetic patients have an increased risk of CHD, considerable attention has been focused on this population group. A study in type II diabetic patients found that vitamin E had anti-inflammatory effects resulting in reduced plasma concentrations of CRP, while vitamin C had no effect on CRP (44).

In a randomized trial on a cohort of passive and active smokers, Block et al (2004) found that the administration of 515 mg/day vitamin C resulted in a 25% reduction in CRP concentration (95% CI: -38.9 % to –5.5%; p=0.036) after adjusting for BMI and baseline CRP levels. However, no significant effects were seen in subjects receiving an antioxidant mixture or placebo. Participants in this study were limited to “healthy” active and passive smokers (both men and women) who consumed less than 4 fruits and vegetables per day. However, because

the analysis was adjusted only for BMI and CRP and not for any other dietary variables, these results may not be reliable.

Food sources of alpha tocopherol (AT) include wheat germ oil, sunflower and peanut oils, margarines, almonds, sunflower seeds, peanuts and peanut butter (45). Numerous studies have failed to show that AT significantly affects inflammation biomarkers. For example, there was no effect on inflammation when subjects were supplemented with 800 IU/d all-rac AT as opposed to the significant inverse association commonly found with the natural RRR-AT isomer (46). Current research has shown that the "natural" form of vitamin E has approximately twice the bioactivity in humans than the synthetic vitamin (45), and it has been suggested that "natural" vitamin E (AT) may protect omega-3 fatty acids from lipid peroxidation (42).

Despite the initially promising hypothesis, observational studies have failed to demonstrate cardio-protective effects of supplemental AT. The GISSI study (1999) randomized men with a recent MI to one of 3 dietary supplement groups: omega-3 fatty acids alone (0.86 mg EPA+DHA), vitamin E alone (300 mg synthetic AT), or a combination of both. This study, as well as others (21), used synthetic AT and did not find a significant relationship between AT supplementation and the risk of a second MI.

Fish is a rich source of selenium, an essential trace element antioxidant. Other dietary sources of selenium include bread, cereal, poultry and meat. Studies of dietary intake of selenium on cardiovascular disease have yielded inconsistent results (47). A secondary analysis in a randomized trial showed no significant associations between daily selenium supplementation (200 ug/day) and cardiovascular disease endpoints during the 7.6 years of follow up. These results suggest that selenium alone may not be cardio-protective, but does not

exclude the possibility that selenium in combination with other foods (such as fish) or nutrients may have cardiovascular benefits.

Anti-inflammatory effects of aspirin and other hypolipidemic drugs

In 1997, the American Heart Association (AHA) issued a statement for healthcare professionals on the therapeutic effects of aspirin in patients with cardiovascular disease (CHD) (48). Aspirin can reduce the risk of occlusive vascular events by inhibiting platelet aggregation. Numerous studies have been conducted with aspirin in high-risk patients (men with history of CHD) but few primary prevention studies on aspirin and CHD risk have been done, especially in women. Two prospective cohort studies reported statistically significant reductions in the risk of a first MI in adult men who consumed aspirin (48, 49). After alternate-day dosage of 325 mg, the risk of a first MI in 22,071 participants in the US physicians health study was reduced significantly by 44%. However, a smaller cohort study in Britain failed to find a significant reduction in CHD risk after aspirin supplementation (48).

In March 2005, the Women's Health Initiative study published findings from a randomized prospective cohort of women aged 45 years and older (50). The women received 100 mg aspirin or placebo every other day and were followed for ten years for a first cardiovascular event. Women who consumed aspirin had a non-significant 9% reduction in risk of a first cardiovascular event (95% CI: 0.8 to 1.03, p=0.13). Aspirin significantly reduced the risk of stroke by 17% in the treatment versus placebo group (RR 0.83, 95% CI: 0.69 to 0.99, $p<0.04$) (51).

In addition to aspirin, other medicines are prescribed to reduce the risk of CHD by lowering blood lipids and CRP. Drugs that are used to lower CRP include statins, glitazones, fibrates, niacin, and fish oil (52). Statins are also used to lower LDL (53), and TC (54). Three

recent studies demonstrated the beneficial effects of statins on blood lipid profiles and CRP. A randomized trial performed between 2000 and 2004 used a two-by-two factorial design to compare the effects of intensive and moderate statin therapy and gatifloxacin versus placebo on the risk of recurrent coronary events after acute coronary syndromes in 3,745 male and female patients (mean age of 58 years). Statin therapy resulted in lower LDL and CRP in patients (p values not provided) (53).

Nissen and colleagues (2005) conducted a similar study in which 502 patients with CHD were randomly assigned to receive either moderate (40 mg/day of prevastatin) or intensive (80 mg/day of atorvastatin) statin treatment over 18 months. Comparison of baseline versus post-treatment CRP, LDL, and TC for the combined sample (moderate and intense drug use) demonstrated significant decreases. LDL concentrations post treatment decreased from 150.2 mg/dL to 94.5 mg/dL ($p<0.001$); CRP concentrations decreased from 2.9 to 2.3 mg/L, $p<0.001$ and TC concentrations decreased from 232 mg/dL to 169.2 mg/dL, $p<0.001$. Treatment resulted in non-significant increases in HDL concentrations (42.6 mg/dL to 43.8 mg/dL, $p=0.11$) (54).

Estrogen and other hormones affect hepatic lipid and lipoprotein metabolism. During menopause, the body decreases the production of estrogen, resulting in significant reductions of circulating levels of estradiol and estrone (55) which adversely affect blood cholesterol and CRP concentrations. Therefore, postmenopausal women have an increased risk of CHD, not only due to advancing age but also because serum levels of TG, TC, and LDL are increased while HDL levels decrease (56). Some postmenopausal women choose to take hormones, i.e. hormone replacement therapy. Several case control studies and clinical trials demonstrated that hormone therapy increased plasma CRP concentrations (28, 55, 57).

Exercise and physical activity

It is generally recognized that habitual physical activity helps to reduce the risk of CHD and alleviate symptoms associated with CHD in diagnosed patients. In particular, physical activity may prevent such risk factors as high blood pressure, insulin resistance, glucose intolerance, elevated TG concentrations, low HDL concentrations, and obesity. In combination with weight reduction, physical activity can decrease LDL and elevate HDL concentrations. The AHA summarized the evidence for the benefits of physical activity in the prevention and treatment of CHD. Data have consistently shown a graded inverse relationship between coronary artery disease rates and levels of physical activity. The magnitude of the effects of exercise is influenced by type, duration, intensity, and frequency of exercise, the extent of weight lost, and individual variation.

Dietary fiber

Diets high in complex carbohydrates and fiber are associated with reduced mortality rates from CHD (58). Epidemiological studies have repeatedly shown the lipid-lowering effects of diets rich in fruits and vegetables, which are good sources of both soluble and insoluble fiber and tend to be low in fat. Pereira et al (2004) analyzed the original data from 10 prospective cohort studies from the United States and Europe to study the association between the risk of CHD and intake of dietary fiber derived from cereal, fruits, and vegetables, as well as whether the fiber was soluble (pectins, gums, and mucilages) or insoluble (hemicellulose, cellulose, and lignin). Data on fiber derived from cereals, fruits, and vegetables were available for all of the studies, but only 2 studies included estimates of insoluble and soluble fiber. The authors noted that there is not a standard method for estimating these fiber types based on food tables, so the results provided for the insoluble and soluble fibers were noted to be exploratory only. In 6 to

10 years of follow-up, 5,249 incident total coronary cases and 2,011 deaths occurred in 91,058 men and 245,186 women. After adjusting for demographics, BMI, and lifestyle factors, each 10g/day increment of total dietary fiber (adjusted for energy and corrected for measurement error) was associated with a 14% decrease in risk of all cardiovascular events (RR=0.86, 95% CI, 0.78 to 0.96) (59).

Several mechanisms have been proposed to explain how fiber reduces the risk of cardiovascular disease, but none have been proven. To ensure nutrition and maximize the cholesterol-lowering impact of a healthy diet, the AHA recommends a total dietary fiber intake of 25 to 30 g/day from foods (not supplements).

Dietary trans-fatty acids

Efforts to estimate the dietary intake of trans fatty acids (or trans fats) in the US are difficult because a complete database for the trans fatty acid content of foods is not available. Therefore, most intake estimates are based on disappearance data, food-questionnaire data or analysis of self-selected diets (60). Clinical data have shown consistently that trans fatty acid intake results in higher plasma cholesterol levels compared to other native oils and saturated fats. Due to the static temporality nature of cross sectional studies and the limited information on trans fat content of foods, epidemiological studies that have investigated the relationship between trans fat intake and CHD risk have yielded inconsistent results (60). The NHANES (1999-2002) did not separate out trans fat from data on other fats obtained during the 24-hour dietary recall interview, so trans fat was not included in the current study.

Alcohol Consumption

Depending on the "dose," alcohol consumption has been shown to be either a protective or harmful variable in determining the risk of heart disease. Observational studies in middle aged or elderly men and women demonstrated a decreased risk of CHD with moderate consumption of alcohol, but this relationship appears to be J-shaped (14). Consuming 1-2 drinks/day was associated with a 30-50% reduction in CHD risk. Based on the total body of evidence available, the AHA concluded that moderate consumption of alcohol does support a true protective effect. Heavy consumption of alcohol as seen in alcoholics, however, appears to adversely affect the heart muscle and arterial tissues. According to the Health, Aging and Body Composition Study of 2,574 healthy elders, moderate drinking (1-7 drinks/week) resulted in lower levels of markers of inflammation (CRP and IL-6) compared to heavy drinking (>7 drinks/week) or no drinking at all (15). Alcohol consumption (g/day) was reported in the NHANES (1999-2002) 24-hour dietary recall interview.

Smoking Status

Tobacco smoke is harmful to overall health. In a 1997 report on cigarette smoking, cardiovascular disease and stroke, the AHA concluded that 30% of deaths due to CHD were attributable to cigarette smoking, and the risk was dose dependent. Cessation of smoking reduces the risk of CHD. Cigarette smoking also is associated with elevated levels of CRP (52) and other inflammatory markers (12). Levels of serum cotinine (a metabolite of nicotine) can be used to evaluate exposure to tobacco (1).

Chapter 2: Methods

Data

The NHANES is a stratified, multistage probability sample of the civilian non-institutionalized U.S. population, which interviews approximately 5,000 people in their homes per survey year. The respondents complete a health examination component in mobile examination centers (MEC). Low-income persons, adolescents 12-19 years, people over 60 years old, African Americans and Mexican Americans are over-sampled (1). Two of the major objectives of the NHANES are to estimate the number and percent of persons in the US with selected diseases and risk factors, and to study the relationship between diet, nutrition and health (1).

Population

The sample population of interest is limited to women ages 16-49 years for whom blood Hg data are available. Variables measured in NHANES are separated by collection year (1999-2000 and 2001-2002), by type of data collection (i.e. demographic, examination, laboratory or questionnaire), and further subdivided by category of variable (e.g. total nutrient intake, cardiovascular health, physical activity). All of the files are linked by the common participant number (variable name SEQN). The methodology for the variable selection and data merging in the current study is as follows:

1) All variables of interest by were merged by collection year, noting that not all survey participants had valid data for the variables of interest.
2) When a person did not have a measured value or did not participate in the portion of the survey of interest, this person's data point showed as "missing".

3) Once the variables were merged for one collection year for the entire NHANES population, the frequencies were checked for completeness in the combined dataset to those published for each variable in the NHANES website.
4) The same methodology, as described in 1 through 3 above, was used for the 2001-2002 NHANES dataset.
5) Once the two separate datasets containing all variables of interest were checked and complete, variables that were the same but which had a slightly different variable name format were merged with a common name. For example, the variable for the highest level of education was coded as "DMD140" in the 1999-2000 dataset and "DMDEDUC" in the 2001-2002 dataset. The 1999-2000 dataset variable was renamed "DMDEDUC" to be consistent with the 2001-2002 variable name.
6) Once the two datasets were combined, the frequencies were double-checked again against those published on the NHANES website.
7) Once all variables were checked, the dataset was abridged to only women aged 16-49 years.

Variables

The NHANES collected demographic, questionnaire and laboratory data on approximately 5,000 people. Markers and risk factors for CVD were all measured in the NHANES MEC. Selected variables such as low density lipoprotein (LDL), triacylglycerols (TG), plasma glucose, dietary supplement intake, and total blood mercury were measured on only a sub-set of the NHANES population while others (e.g., hair mercury) were collected for only two of the four survey years. The variable selection for the current analysis was restricted to data availability in the combined 1999 to 2002 NHANES data releases.

Fish intake

24-Hour Dietary Recall

Data Availability in the NHANES (1999-2002)

24-hour dietary records were collected by in-person interviews using a computer-assisted dietary interview software program that was developed for the NHANES. Charts and diagrams were provided to the respondents to aid accuracy of recall. In the case of an incomplete dietary recall, the interviewers followed up with the respondent by telephone. All survey participants were eligible for the dietary recall as translators and proxy reporting (parents or guardians) were both allowed. Subjects were excluded only if cognitive difficulties prevented the dietary recall or if proxies were unavailable. The dietary intakes of nutrients from reported foods were calculated using the USDA food and nutrient database version 1.0 for the 2001 to 2002 dataset. Nutrients were calculated in the 1999-2000 dataset using the USDA 1994-1998 survey nutrient database. The overall acceptability of each dietary recall was evaluated and assessed for reliability. If less than 25% of the foods reported had missing descriptive information, less than 15% of the foods reported had missing amounts, or any meal reported did not have at least one identified food the recall data was labeled as unreliable. A second day of diet recall was collected in the 2002 NHANES by telephone interview; however, these data were not released to the public because of confidentiality issues.

Use of 24-Hour Dietary Recall Data in the Current Analysis

Respondents with dietary recall data coded as unreliable were excluded from the current analysis (variable DRDDRSTZ). Because omega-3 fatty acids are derived from both plant and

animal sources, a new variable was created to represent marine derived omega-3 fatty acids (DHA+EPA). The variables for the omega-3 fatty acids DHA (20:5) and EPA (22:6) were summed to represent DHA + EPA from food sources and included as a single continuous variable in the final regression models.

30-Day Fish Food Frequency Questionnaire

Data Availability in the NHANES (1999-2002)

The NHANES (1999-2002) provided a 30-day food frequency questionnaire (FFQ) for 31 varieties of fish and shellfish. Only children 1-5 years old and women 16-49 years old completed this questionnaire.

Use of 30-Day Fish FFQ in the Current Analysis

Total fish meals consumed over 30-days were determined by combining responses from each of the individual fish 30-day FFQ. Total frequency of fish intake was not normally distributed. Because it is count data, a square root transformation was applied to achieve normality. The data for total frequency of fish consumed were split into four categories (none, 1-4 times/30 days, 5-8 times/30 days, and 9+ times/30 days) to investigate the descriptive statistics. These groupings were selected to match the AHA recommendations of eating fish twice weekly, which is approximately 8 times per month. The square root of total frequency of fish consumption was included as a continuous independent variable in the regression models.

Omega-3 Fatty Acid Dietary Supplements

Dietary supplement use information was collected on a subset of NHANES participants. A preliminary review of the NHANES 1999-2000 dataset demonstrated that less than 1% of the

entire US population reported consuming a dietary supplement containing fish oil. Based on these findings, fish oil supplementation was not included in the current analysis.

Assessing omega-3 fatty acid and fish intake

Researchers have used a variety of methods to assess intake of omega-3 fatty acids and fish. Omega-3 fatty acid concentrations in both plasma and adipose tissue are good biomarkers of dietary omega-3 fatty acid intake (61, 62). Anderson and colleagues (1999) found a slightly stronger correlation for DHA between dietary intake and plasma levels (r=0.52) compared to adipose tissue levels (r=0.49) (61). However, adipose tissue levels more accurately reflect long-term dietary intake (in the absence of recent weight loss) (63). Plasma levels are a reliable measurement for recent (days to weeks) food intake (63) and are shown to be the most sensitive indicators of changes in polyunsaturated fatty acids. Unfortunately plasma and adipose tissue measurements were not collected in NHANES (1999-2002).

Mercury exposure

Assessing Exposure to mercury

Previous studies have used hair, toenail and whole blood to assess environmental exposure to Hg (2, 5, 35, 38, 40, 64, 65). Hair Hg levels have been used historically as a biomarker of chronic exposure (36, 66); however hair Hg represents both environmental and dietary exposure to Hg. Toenail Hg has not yet been established as a reliable biomarker; however, was used in multiple studies. Toenail Hg has not yet been established as a reliable biomarker but has been used in multiple studies. Measurements of total blood Hg levels may not capture long-term exposure to Hg as accurately as hair levels. However, since the half-life of Hg is approximately 70 days, measuring levels in blood should accurately represent the Hg

intake from fish as indicated by the 30-day fish FFQ, and possibly the Hg exposure from fish consumed prior to this FFQ.

Data Availability in the NHANES (1999-2002)

The NHANES 1999-2002 included measurements of blood, hair, and urine Hg (total and inorganic) for women of childbearing age (16-49 years of age) in an attempt to determine the exposure to Hg from fish. Blood and hair measures were also included for children (1-5 years of age). Hair Hg concentrations were obtained for the NHANES 1999-2000 only and were not included in the current study. Toenail Hg was not measured in the NHANES.

Total blood mercury: Total Hg in whole blood was measured in the NHANES (1999-2002) using the methods described by T. Guo and J. Bassner (1). Flow injection cold vapor atomic absorption analysis was conducted with on-line microwave digestion.

Total hair mercury: The NCHS collected hair samples from women ages 16-49 and children ages 1-5. A 3 cm segment of hair was removed from the occipital region of the scalp and used to characterize recent exposure to MeHg over a relatively uniform time interval (2.5 months). Approximately 100 mg (or 100 strands) of hair were collected from eligible participants. Details of the Hg analysis are described in McDowell et al, 2004. Data on hair Hg are available only for the 1999-2000 NHANES dataset.

Total urinary mercury: Urinary Hg was measured in women (16-49 years of age) and children (1-5 years of age) in the NHANES 1999-2000. Urinary Hg reflects exposure to inorganic Hg, which is not of interest in the current study.

Use of Mercury Data in the Current Analysis

Because limited data on hair Hg concentrations were available, the current study used total blood Hg data as the dependent variable to represent exposure to MeHg from fish. The Shapiro Wilk test was used to test for normality. These data were log transformed to achieve normality.

Risk markers of CHD

Cholesterol (Total, HDL):

Data Availability in the NHANES (1999-2002)

Blood lipid profiles including TC and HDL were collected in NHANES (1999-2002) participants older than three years of age. TC was determined enzymatically in serum or plasma. The NHANES (1999-2002) documentation provides information on the laboratory assays and methodology used to measure blood lipids concentrations.

Use of Blood Lipids Data in the Current Analysis

TC and HDL were both included as continuous dependent variables in separate regression analyses. Both variables were normally distributed using the Shapiro Wilk test, so the data were not transformed.

Triacylglycerol (TG)

Data Availability in the NHANES (1999-2002)

Data for TG were available only for NHANES (1999-2002) participants aged 3 years and older who did not meet any exclusion criteria and who were examined in the morning sessions only (i.e. a sub-sample). TG was measured enzymatically in serum or plasma using a series of coupled reactions (1).

Use of TG in the Current Study

Despite the limited number of women for whom TG data were available; a separate regression analysis was performed to determine the relationship between fish consumption (30 day fish FFQ) or DHA+EPA intake on TG. This analysis was limited to 577 women with valid data for the variables of interest.

LDL Cholesterol

Data Availability in NHANES (1999-2002)

LDL was calculated in NHANES (1999-2002) using the Friedewald calculation:

[LDL cholesterol] = [TC] – [HDL cholesterol] – [TG/5]

Because TG was measured for morning participants only, the calculations for LDL were limited to those participants with valid TG data.

Use of LDL in the Current Study

Despite the limited number of women having calculated LDL data; a separate regression analysis was performed to determine the relationship between fish consumption (30 day fish FFQ) or DHA+EPA intake on LDL. This analysis was limited to 577 women with valid data for the variables of interest.

C-reactive protein

Data Availability in the NHANES (1999-2002)

There are two methods of measuring CRP. The newest technique is called high sensitivity CRP [(hs) CRP] and the second technique is simply called CRP. According to the AHA (hs) CRP is measured for seemingly healthy people to determine their risk of CHD and

ranges from 0.5 -10 mg/L. The non-high sensitivity CRP test (levels range from 10-1000 mg/L) is ordered for patients at risk for bacterial or viral infection (such as occurs following surgery) or for patients with chronic inflammatory.

According to the NHANES (1999-2002) documentation, the NHANES researchers used latex-enhanced nephelometry with a Behring Nephelometer to quantitatively determine CRP concentrations. CRP concentrations were calculated by using a calibration curve.

Use of CRP in the Current Study

The CRP data for women ages 16 to 49 were not normally distributed so a log transformation was needed to achieve normality. CRP was treated as a continuous dependent variable in the multiple regression analyses.

Reactive Oxygen Species

Chronic and acute overproduction of reactive oxygen species (ROS) under pathophysiologic conditions is integral in the development of CHD. The role of ROS in atherosclerosis has been demonstrated in animal models but clinical trials have not provided convincing evidence that antioxidants decrease the risks of CHD morbidity or mortality (67). This may be due to a lack of specific and sensitive biomarkers for assessing the oxidative stress phenotypes underlying CHD. Additional research is necessary to identify reliable biomarkers of antioxidants and oxidative stress (67).

Variables other than mercury and omega-3 fatty acids known to affect CHD

Age and BMI

Data Availability in the NHANES (1999-2002)

Age and BMI were reported in the demographic portion of the NHANES (1999-2002). Age was reported in years for women aged 16-49 years and BMI was calculated as weight (kg) divided by height squared (m^2). NHANES participants were weighed and their standing height was measured in the MEC by trained technicians.

Use of Age and BMI in the Current Study

Age of subjects was stratified by decade for the regression models. Women aged 16-19 years were used as the reference population for comparisons between age groups. BMI was included as a continuous variable in the regression models 2 and 3.

Ethnicity and Education

Data Availability in NHANES (1999-2002)

Self reported ethnicity and education were recorded in the NHANES. There were five ethnicity categories including Non-Hispanic white, non-Hispanic black, Mexican American, Other Hispanic and Other. The NHANES documentation indicated that "other" race includes multiracial, those with a single racial/ethnic identity other than the four listed above, those who reported more than one racial identity and those with missing values on race/ethnicity. Level of education was coded as three categories: less than high school or GED, high school or GED, more than high school.

Use of Ethnicity and Education Variables in the Current Study

To increase the sample size of each ethnic group, the "other Hispanic" and "other" groups were merged into one ethnicity category. Non-Hispanic whites were used as the

reference population in the current study. Ethnicity was included as an indicator variable in the regression analyses.

Since a majority (40%) of subjects in the sample had an education level of "less than high school," this group was used as the reference population for education. The level of educational attainment was included as an indicator variable in the regression analyses.

Diabetes, arthritis, and pregnancy

Data Availability in the NHANES (1999-2002)

Diabetes is known to increase the risk of CHD. NHANES participants ages 12 and above provided measurements of plasma glucose, insulin, c-peptide and glycohemoglobin, and self-reported if a doctor had previously diagnosed them with diabetes (NHANES variable name, DIQ010). The plasma glucose concentrations were measured on a sub-sample of the NHANES population.

Arthritis increases inflammation and results in elevated CRP concentrations. The presence of arthritis was listed as a current medical condition in the in-person questionnaire if respondents indicated that their "doctor ever said you had arthritis" (MCQ150A).

Pregnancy testing was conducted using urine and blood specimens in women aged 12-59 years in the NHANES (1999-2002). Initial urine tests were conducted and positive pregnancy tests were confirmed with serum pregnancy tests using the Icon 25 hCG (Urine/Serum) test kit. This assay uses a combination of monoclonal and polyclonal antibodies to detect elevated levels of hCG in urine or serum, an indication of pregnancy (URXPREG). All samples were tested in the NHANES MEC. In addition, females ages 12+ self-reported their pregnancy status in the in-person interview (RHQ_141).

Use of Self-Reported Diabetic, Arthritic, and Pregnancy Variables in the Current Study

Only 1,747 out of 4,084 women aged 16-49 years had valid plasma glucose data (prior to excluding women with missing data for other variables of interest). Therefore, the self reported diabetic status questionnaire response was used to identify persons with diagnosed diabetes. Participants with self-reported diabetic status (yes) were excluded from the analysis (n=104). A sensitivity analysis was used to check if subjects who self-reported being non-diabetics actually had plasma glucose concentrations in the normal range.

Only 227 of 1827 women with valid data indicated self-reported arthritis. This variable was an insignificant explanatory variable when tested in three multiple regression models (CRP, HDL and TC), therefore, it was excluded from the final analysis.

Self reported pregnancy status was used in the current analysis. Of all women aged 16-49 years, 674 reported being pregnant. These women were excluded from the analyses.

Medical history of coronary heart disease

Data Availability in the NHANES (1999-2002)

An in person cardiovascular disease questionnaire (CDQ_B) was used in the NHANES (1999-2002) to collect data on symptoms known to be associated with angina pectoris or respiratory disease. The current physician-diagnosed medical conditions that subjects were asked to report included congestive heart failure (MCQ160B), coronary heart disease (MCQ160C), or angina (MCQ150D), or had a heart attack (MCQ160E), or stroke (MCQ160F).

Use of Self Reported-Current Medical Condition Variables in the Current Study

Because so few women (n=27) answered “yes” to the individual current medical questions relating to cardiovascular medical history, a new variable was created to represent

"yes" to any of the above five conditions (congestive heart failure, coronary heart disease, angina, heart attack or stroke). Self reported cardiovascular medical history was not a significant explanatory variable in any of the models (CRP, HDL, TC), and was excluded from the final regression models.

Antioxidant intakes (vitamin C, vitamin E, and selenium)

Data Availability in the NHANES (1999-2002)

Antioxidant intake was extrapolated from the foods reportedly consumed in the 24-hour dietary recall interview. Antioxidant levels in foods were determined by the National Centers for Health Statistics (NCHS) using the United States Department of Agriculture nutrient database (USDA/ARS, 2003). Serum concentrations of vitamins A, E (AT), two retinyl esters and carotenoids were measured in NHANES using high performance liquid chromatography and multiwavelength detection. However, antioxidant status in this analysis was based on food consumption reported in the 24-hour dietary recall, which may be a better estimate of daily nutrient intakes.

Use of Antioxidant Intake Variables in the Current Study

All antioxidants were included in the regression models as continuous independent variables.

Aspirin and hypolipidemic prescription medications

Data Availability in NHANES (1999-2002)

The prescription medication and medication sub-sample provides personal interview data on the use of these products during a one-month period prior to the survey date. During the in-person interview, respondents either reported the name and duration of use of their prescription medication or dietary supplement, and if available, they provided the physical containers for these products. The reporter entered the medication name into a database containing all prescription and non-prescription drug products available in the US (Master Drug Database, proprietary database of Facts and Comparison). Each item was then identified as an "exact" or "most similar match," or else it was designated as a "drug not found on list." All reported medication names were converted to their standard generic ingredient names (e.g. acetaminophen).

Use of Prescription Medicine (Lipid Altering and Inflammation Altering) Variables in the Current Study

An initial analysis was conducted to determine the prevalence of prescription medication use in the study sample. 1,108 of 2,209 women indicated taking prescription medication, but not all of the drugs taken affect markers of CHD or inflammation. There was a significant positive association between prescription drug medication and all three dependent variables (CRP, HDL, TC). Therefore, we used the Medline Plus for Drugs and Supplements database to look up each of the generic ingredients reported in the prescription medication questionnaire. Only drugs (hormones, antibiotics, anti-inflammatory and anti-virals) affecting lipid profiles were included in the analysis (see Appendix 1 and 2 for a listing of drug ingredients included). Two new variables were created to indicate participants who reported taking drugs containing

lipid or inflammatory altering ingredients. The two separate prescription drug variables were included in regression models as categorical (indicator) variables.

Hormones prescription medications

Prescription drugs containing hormones were included in the inflammatory prescription drug variable. 785 of 2,209 women reported taking a prescription drug known to affect inflammation, which include drugs containing hormones.

The NHANES questioned female participants (ages 12 years and older) in a private face-to-face interview on reproductive health (RHQ_B). The questionnaire contained questions on menstrual history, pregnancy history, lactation, oral contraceptive and hormone replacement therapy use, and other related conditions.

Serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were collected for women aged 35-60 years as well as questionnaire data on menstrual history to classify women according to menopausal status. 117 of 1,914 women aged 16-49 years with valid data for the current analysis were menopausal. This variable for hormone concentrations was excluded from the final regression model since any hormones being ingested were included in the prescription medication portion of the analysis and age was a control variable.

Exercise and physical activity

Data Availability in the NHANES (1999-2002)

The NHANES (2000-2001) included a physical activity questionnaire (PAQ) that asked questions related to daily- leisure-time- and sedentary-activities. Respondents aged 16 years and older were asked these questions during the in-person household interview. Some of the data were recoded by NCHS due to inconsistency. For example, respondents who answered yes

to vigorous activities (PAD200) but did not report any vigorous activities or reported a vigorous activity for less than a 10-minute duration were recoded as “no”. A similar adjustment was done for moderate activities (PAD320). Frequency of physical activities involving tasks and yard work (PAD120) and muscle strengthening activities (PAD460) were reported by the respondents on a daily, weekly, or monthly basis. These data were then converted to a 30-day frequency. Implausible responses (e.g. 12 hours or more per day) were recoded to “missing”.

In addition to physical activity the survey assessed cardiovascular fitness by measuring maximal oxygen uptake ($V0_2$ Max testing). This sub-max test was prescribed based on gender, age, BMI, and self-reported level of physical activity. Participants were assigned to one of eight treadmill protocols. The overall aim of the treadmill test was to elicit a heart rate approximately 75 percent of the age predicted maximum (calculated by subtracting the age from 200) by the end of the test. Each test included a two-minute warm-up, two 3-minute exercise stages, and a two-minute cool down. Heart rate was monitored using an automated monitor with four electrodes connected to the subject (thorax and abdomen). Blood pressure and perceived exertion (Borg Scale) were measured at the end of each exercise phase.

The outcome variable of interest for this test (CVDESVO2) is the estimated maximal oxygen uptake (ml/kg/min). This value was extrapolated using measured heart rate responses to known levels of exercise workloads, assuming the relation between heart rate and oxygen consumption is linear during exercise. This concrete variable is less prone to self-reporting errors commonly seen in questions related to physical activity.

Use of Physical Activity in the Current Study

Very few women aged 16-49 years (n=1,790) completed the required testing for estimated maximal oxygen uptake prior to excluding women who had missing data for other essential variables in the analysis (i.e. blood lipids, total blood mercury, fish FFQ). This limited the sample for analysis by 50%, so self reported physical activity was used instead of VO2 max. Participants selected one of eight coded variables (see below) that best described their physical activity status.

(0) Little or no regular recreation, sport or physical activity and avoids walking or exertion
(1) Little or no regular recreation, sport or physical activity but walks for pleasure and occasionally exercise
(2) Participating regularly in recreation or work requiring modes physical activity for 10 to 60 minutes per week.
(3) Participating regularly in recreation or work requiring modest physical activity for more than 60 minutes per week.
(4) Participating regularly in heavy physical activity for less than 30 minutes per week.
(5) Participating regularly in heavy physical activity for 30-60 minutes per week.
(6) Participating regularly in heavy physical activity for 1-3 hours per week.
(7) Participating regularly in heavy physical activity for more than 3 hours per week.

Since few participants reported exercise in the upper and lower ranges, we condensed the scale to three categories: "low," "moderate," and "high." Low included NHANES categories 0-1, "moderate" included categories 2-3 and "high" included categories 4-7. The majority of subjects reported "moderate" physical activity. The data were included in all regression models as a categorical variable, with "low" exercise as the reference population.

Macronutrients (Carbohydrate, protein, fiber, fat, saturated fat, polyunsaturated fat, mono-unsaturated fat, trans fat)

Data Availability in the NHANES (1999-2002)

Similar to the antioxidants mentioned earlier, energy, carbohydrate, dietary fiber, protein, and fat (monounsaturated, polyunsaturated, and saturated fatty acid) intakes were obtained from the foods reportedly consumed in the 24-hour recall data. The nutrient content of foods was determined by the National Centers for Health Statistics (NCHS) using the United States Department of Agriculture nutrient database (USDA/ARS, 2003).

The NHANES (1999-2002) did not separate out the varieties of dietary fiber. Dietary fiber was reported as a single continuous variable derived from the 24-hour dietary recall interview and was included in the current study. Along with fiber, the macro and micronutrients were included in all regression models as continuous variables. However, because initial analyses revealed the existence of collinearity between most nutrients and the three dependent variables of interest (LDL, HDL, TC), only fiber, monounsaturated fat, and saturated fat were included in our regression models as potential confounding variables.

Alcohol consumption

Data Availability in the NHANES (1999-2002)

The NHANES (1999-2002) obtained alcohol intake (including beer, wine, wine coolers and liquor) but excluded sips consumed for religious purposes. Two questions were asked via Audio computer Assisted Personal Self Interview to respondents aged 12-19 years:

1. "During the past 30 days, on how many days did you have at least one drink of alcohol?" Responses range from 0, 1-2, 3-5, 6-19, 20-29, all 30 days, refused, and don't know.

2. "How many days did you have 5 or more drinks of alcohol in a row, that is, within a couple of hours." This question (ALQ.040) also addressed alcohol consumption during the past 30 days.

Information about alcohol consumption for adults aged 20+ also was recorded by Computer Assisted Personal interview (CAPI). The NHANES variable ALQ.120 separated drinkers from non-drinkers in the past year. ALQ.110 indicated whether or not the respondent ever consumed alcohol. ALQ.120 asked how often the subject drank any type of alcoholic beverage "in the past 12 months". In addition to the CAPI, alcohol consumption was recorded in the 24-hour dietary recall interview.

Use of Alcohol Consumption in the Current Analysis

All alcohol-containing beverages reported consumed in the 24-hour dietary recall interview were included in the regression analyses as a continuous variable.

Smoking status

Data Availability in NHANES (1999-2002)

Environmental exposure to smoke can cause an increase in inflammation. Tobacco use has been associated with multiple cancers as well as CHD among smokers (1). The tobacco component of the NHANES included questionnaire items on current and past use of cigarettes, pipes, cigars, and smokeless tobacco. It also addressed exposure to environmental tobacco smoke at home and work, as well as the use of nicotine replacement products (patch, gum). Cotinine (a metabolite of nicotine) was collected as a biochemical marker of exposure in participants ages 3 and older (1).

Use of Smoking Status in the Current Analysis

A review of the self-reported tobacco use questionnaire resulted in few (n=715) women who actually reported smoking at least 100 cigarettes per year, severely restricting the sample size for analysis. Because smoking is known to be harmful to health, some women may be reluctant to admit to occasional smoking. In addition, the self-reported smoking status will not identify women who are exposed to second hand smoke. Serum cotinine status was used as a biomarker of nicotine exposure, which was collected for the majority of our sample of women aged 16-49 years. In the current study, serum cotinine was included as a continuous control variable in all regression analyses.

Statistical methods

Univariate analyses and descriptive statistics were conducted on all variables to view the distribution of each variable as well as check the association of potential confounding variables with each dependent variable. The distributions of continuous variables were tested for normality using the Shapiro Wilk test and supported by visual evaluation of histograms. Data transformations were conducted, when necessary, to transform the variables to normality (the sum of 30-day fish food frequency (square root), CRP (log), and total blood Hg (log)).

A small subset of women aged 16-49 years (n=211) were eliminated from the analysis sample due to missing four-year exam statistical weights. Of the remaining women, data were statistically weighted using the four-year statistical weights (WTMEC4YR). To correctly calculate the standard errors, the PSU (SDMVPSU) and Strata variables (SDMVSTRA) were used to account for the survey design effect of the NHANES (1999-2002).

Means and standard errors were calculated using the *survey* commands in STATA for women ages 16-49 who were not pregnant, did not have diabetes (self reported) and had valid 24-hour dietary recall data. The mean of each continuous descriptive variable for the three categories of fish consumers (1-4 times per 30 days, 5-8 times per 30 days and $\geq$ 9 times per thirty days) was compared to the mean for non-fish consumers using the accumulated Adjusted Wald Test.

Outliers for total fish consumption were included in the analysis. Sensitivity tests were conducted for all regression models excluding the outlier points (total fish frequency per 30 days=118, total blood mercury= 4.0 ug/L). This analysis did not produce significantly different results than those presented in the current study.

A total of 10 separate multiple linear regression analyses were conducted (LDL, HDL, TC, TG and CRP each on both DHA+EPA and total frequency of fish consumption, separately). Each regression analysis was split into three tiers. The first tier regressed blood lipids or CRP on total frequency of fish consumption or DHA + EPA controlling for only age (by decade) and BMI (continuous). The second model controlled for all variables noted above including (age groups (20-29, 30-39, 40-49) BMI, self reported educational attainment, self reported ethnicity, self-reported physical activity, anti-inflammatory prescription drug use, lipid altering prescription drug use, serum cotinine, vitamin C, selenium, fiber, alcohol, mono-unsaturated fatty acids, saturated fatty acids). Some of the continuous variables were re-coded into categorical variables to better understand the relationships between independent and dependent variables (age, DHA + EPA, total fish frequency consumption per 30 days). Model 3 included total blood Hg (log) as a continuous variable. The full model was used in each of the final results presentation.

Chapter 3: Results

Relationship between fish intake, omega-3 fatty acids, mercury, and risk markers of coronary heart disease (NHANES 1999-2002)

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Abstract

Background: Fish consumption has been shown to be inversely associated with coronary heart disease (CHD), which may be due to omega-3 fatty acids. The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are naturally found only in marine sources. Dietary intakes of methyl mercury from certain fish have been hypothesized to increase the risk of CHD.

Objective: Investigate the relationship between 30-day fish food frequency (FFQ), DHA+EPA intake, total blood mercury concentrations, and risk markers of CHD in women aged 16- 49 years (NHANES 1999-2002).

Design: Multiple linear regressions were used to test 1) the relationships between 30-day fish FFQ and DHA+EPA intakes and five CHD risk markers: high density lipoprotein (HDL), low density lipoprotein (LDL) and total cholesterol (TC), triacylglycerol (TG), and C reactive protein (CRP) and 2) if total blood mercury attenuated any cardio-protective benefits.

Results: Total 30-day FFQ was negatively associated with CRP (95% CI: -0.19 to -0.02, p=0.015) and positively associated with HDL (95% CI: 0.31 to 2.5, p=0.014). Adjustment for other risk factors did not significantly attenuate the associations. 30-day fish FFQ and HDL were not significantly associated when total blood Hg was included in the model (95% CI: -0.60 to 1.6, $p<0.05$) which was due to collinearity between fish intake and total blood mercury.

Conclusions: Mercury concentrations in fish are not high enough to significantly impact CHD risk in women 16-49 years. The levels of DHA+EPA and other nutrients in fish may be

adequate to offset the hypothesized risks of ingesting mercury in fish. Women can consume fatty fish in moderation as a part of a healthy diet.

KEY WORDS: Fish, Mercury, NHANES, CHD, CRP, dietary exposure

Introduction

Fish and fish oils containing the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been shown to have cardio-protective attributes in both healthy individuals and those at high risk of coronary heart disease (CHD) (68). The chief dietary sources of DHA and EPA are fatty fish such as mackerel, lake trout, herring, tuna, sardines and salmon as well as dietary supplements. Consumption of DHA and EPA and fish containing these fatty acids has been shown to reduce blood lipid concentrations of low density lipoprotein cholesterol (LDL), total cholesterol (TC) and triacylglycerol (TG) and to raise concentrations of high density lipoprotein cholesterol (HDL). Omega-3 fatty acid intake is also associated with a reduction in plasma biomarkers of inflammation such as C-reactive protein (CRP) (28). The physiological effects of DHA and EPA include lowering plasma TG concentrations, inhibiting plaque formation, decreasing platelet aggregation and reducing arrhythmias (30).

Fish also contains methyl mercury (MeHg), an environmental contaminant derived from industrial waste and the burning of fossil fuels. MeHg makes its way into streams, lakes and oceans where it becomes incorporated into the food chain of marine animals (32). There is evidence that MeHg may increase the risk of CHD by promoting the formation of free radicals and compromising the function of antioxidants that neutralize these agents (5). Therefore, it is possible that consuming fish contaminated with MeHg may actually increase the risk of CHD.

The hypothesis that MeHg attenuates the cardio-protective benefits of fish consumption remains unproven. A review of seven epidemiological studies conducted to date (5 in men, 1 in women, and 1 with both men and women) suggests an association between MeHg exposure and greater CHD risk, including acute myocardial infarction, but no studies have addressed the

specific role of MeHg on risk markers of CHD (3-6, 69). The objective of the current study is to investigate relationships between 30-day frequency of fish intake, 24-hour DHA+EPA intake, total blood mercury (Hg) concentrations, and risk markers of CHD in women aged 16-49 years who participated in NHANES 1999-2002. Indicators of CHD risk examined include blood concentrations of TC, LDL, HDL, TG, and the inflammatory marker CRP.

Materials and Methods

The NHANES (1999-2002) database was used to investigate relationships between fish consumption, dietary intake of marine omega-3 fatty acids (DHA+EPA), total blood Hg, and risk markers of CHD in women aged 16-49 years. The NHANES is a stratified, multistage probability sample of the civilian non-institutionalized U.S. population which interviews approximately 5,000 people per survey year. Respondents are interviewed in their homes and also complete a health examination component in mobile examination centers (MEC). Low-income persons, adolescents 12-19 years of age, people >60 years of age, African-Americans and Mexican-Americans are over-sampled (1).

Independent Variables

Fish intakes by women aged 16-49 years was assessed as the total number of fish meals reported consumed in a 30-day fish food frequency questionnaire (FFQ) in the NHANES (1999-2002). The NHANES participants did not provide data on the portion sizes of fish reported consumed in the fish FFQ. In this analysis, total fish frequency of consumption data for women aged 16-49 years were grouped into four categories: 0, 1-4, 5-8, or $\geq$ 9 times/30 days. Shellfish were omitted from the analysis because they are low in Hg. For individuals in each of the 4 fish consumption categories, mean blood lipids, CRP, total blood Hg, and nutrient concentrations along with descriptive statistics for selected demographic variables were calculated. The summary statistics were derived using the 4-year statistical weights and adjusting for the statistical design of the survey as recommended by the NHANES analytic guidelines (1).

Fish oil intake was calculated as the sum of DHA+EPA according to data derived from the 24-hour dietary recall interview conducted by the National Centers for Health Statistics.

Fish oil consumption was included in the analysis as a secondary indicator of fish consumption for women aged 16-49 years.

Dietary exposure to Hg was evaluated in this study using total blood Hg concentrations measured in NHANES (1999-2002) female participants aged 16-49 years.

Dependent Variables

The dependent variables of interest in this study were TC, HDL, and CRP. Because TG data were available for a limited number of participants in the NHANES (1999-2002), a separate sensitivity analysis was conducted using serum TG and LDL concentrations.

Covariate Variables

Covariates were included in the regression models to take into account demographic, lifestyle, and nutritional factors. Demographic and lifestyle variables were self-reported unless otherwise noted and included age (grouped for this analysis into 4 categories: 16-19, 20-29, 30-39 and 40-49 years), ethnicity (non Hispanic white, non Hispanic black, Mexican American, other and other non-Hispanic), BMI (kg/m^2, calculated by trained NCHS technicians), educational attainment (less than high school, high school or GED, or greater than high school), physical activity (separated into low, moderate and heavy levels of daily activity), prescription drug use (anti inflammatory or lipid lowering), serum cotinine concentrations (a measure of exposure to tobacco) and medical history of heart disease (physician- diagnosed diseases). The diagnosis of heart disease was classified as congestive heart failure, coronary heart disease, angina, heart attack, or stroke. Intake of individual nutrients was derived from the 24-hour dietary recall interview and was based on NCHS calculations. Nutrient intakes (energy, fiber, monounsaturated fatty acids, saturated fatty acids, total cholesterol, vitamin C, alcohol and selenium) were treated as continuous variables in all models. Nutrients that were omitted from

the models because they were not significantly associated with the dependent variables or caused collinearity included protein, carbohydrate, total fat, and total polyunsaturated fatty acids.

Cohort for analysis

The sample for this study was limited to women for whom data for the independent and dependent variables of interest were available. The NHANES (1999-2002) dataset contains a total of 10,790 women, including 4,084 women aged 16-49 years. Our sample was further restricted to women having measurements for total blood Hg (n=3,637), CRP (n=3,608), 24-hour dietary recall interview (n=3,458), 30-day fish FFQ (n=3,456), TC (n=3,434), and HDL (n=3,435). LDL and TG data were collected on only a subset of women, reducing the sample size to 1,726 prior to testing for confounding variables.

Women were excluded from all analyses if they reported on the demographic questionnaire that they were pregnant (n = 704), if a doctor told them they were diabetic (n = 104), or if their 24-hour dietary recall interview was coded by NCHS as unreliable (n = 207). This occurred if $\geq$25% of foods reported consumed had missing descriptive information, $\geq$15% of foods had missing quantities eaten, or if a meal did not include at least one identified food. In addition, 211 women who did not have 4-year statistical weights were excluded. The numbers excluded are not mutually exclusive. After these adjustments were made, the sample size was reduced to 1,245 women for the analysis of the major dependent variables, while 577 of the 1,245 eligible women were included in the sensitivity analyses for LDL and TG.

Statistical Methods

STATA V. 7.0 (70) was used to conduct all statistical analyses including the univariate summaries and multiple regression models. All estimates were derived using the 4-year MEC statistical weights and adjusting for survey design. Regression analyses were conducted using

the *survey* regression commands in STATA. The distributions of continuous variables were tested for normality using the Shapiro-Wilk test and data were transformed to achieve normality when necessary. Prior to analysis, log transformations were done on CRP and total blood Hg values, and a square root transformation was performed on the total reported 30-day fish food frequency values.

The following hypotheses were tested:

(1) Fish intake is negatively associated with TC and CRP concentrations, and positively associated with HDL and total blood Hg concentrations

(2) DHA+EPA intake is negatively associated with TC and CRP concentrations and positively associated with HDL and total blood Hg concentrations

(3) Total blood Hg will attenuate the cardio-protective benefits demonstrated by fish and DHA+EPA consumption.

The sample was stratified into categories defined by total frequency of fish consumption in the past 30 days (0, 1-4, 5-8, > 9 times/30 days). Means of all continuous variables for each of the 3 fish-consuming groups were compared to the non-fish consuming group using the Adjusted Wald Test.

The associations between total frequency of fish consumption in the past 30-days, blood lipids, and CRP concentrations were tested using multiple linear regression analyses. The initial models (Model 1) adjusted for energy intake and age (separated by decade with women aged 16 -19 years serving as the reference population). To adjust for potential confounding, subsequent multivariate models included known CHD risk factors.

Specifically, Model 2 adjusted for the variables in Model 1 as well as for lifestyle, dietary habits, and medical history of arthritis and CHD. Model 2 also included adjustments for

prescription drug use (anti inflammatory or lipid lowering), ethnicity, educational attainment (indicator variables) and continuous variables such as BMI, smoking (measured as serum cotinine), and 24-hour dietary recall interview intakes of total calories, fiber, saturated fat, monounsaturated fat, vitamin C, selenium, and alcohol (continuous variables).

Model 3 included total blood Hg concentrations (log) in addition to the variables included in Model 2. The analyses were conducted separately for each of the blood lipids and CRP. Similar analyses were conducted for the associations between 24-hour dietary intakes of DHA+EPA (replacing fish consumption frequency) and blood lipids and CRP. Models 1 and 2 provide data for testing for the first two hypotheses relating to the cardio-protective effects of fish consumption and DHA+EPA intake, while Model 3 addresses the third hypothesis (that total blood Hg will attenuate the potential protective effect of fish consumption tested for the first two models). The sample for all multiple linear regression analyses was limited to women aged 16-49 years with valid data for all variables included in Model 3 (n=1,245).

Results

Characteristics of the population

Demographic and lifestyle characteristics, nutrient intake, and blood biomarkers of CHD risk by fish consumption category are summarized in Tables 1-3. The 1,245 non-pregnant women that constitute this study sample had an average age of 32.5 $\pm$ 0.4 years and an average BMI of 26.3 $\pm$ 0.22 kg/m^2. The reported average daily caloric intake of the sample was slightly less than 2,000 calories/day (1993.6 $\pm$ 25.5 kcals/day). The study sample reported an average of 2.9$\pm$ 0.13 fish meals over 30 days (less than one fish meal per week), and had an average intake of 0.1 $\pm$ 0.01 g DHA+ EPA calculated from the 24-hour dietary recall interview, confirming a low fish intake.

Comment [E2]: Dr. Sahoun's notes are not clear, include or exclude this statement? That's fine

The majority of the sample of women aged 16-49 years consumed fish 1-4 times/30 days. When separated by ethnicity the majority of all race/ethnicity groups were split between non-fish consumers and consuming fish 1-4 times/30 days. Study participants who classified themselves as "other Hispanic" and "other" had the greatest percentage of regular fish consumers (fish $\geq$ 9 times/30 days) (10%). The majority of Mexican Americans were non-fish consumers (43%) and light (1-4 times/30 days) fish consumers (48%).

Regular fish consumers ($\geq$9 times/30 days) had the lowest serum cotinine levels than all of the other fish and non-fish consuming groups. Fish consumers had significantly lower serum cotinine levels than non-fish consumers ($p=0.0043$), suggesting that fish consumers are less likely to smoke and to be exposed to second hand smoke (Table 1). Reported fish consumption patterns were similar between the three physical activity groups. The majority of women in all physical activity groups were non-fish consumers (26-29%) and light fish consumers (1-4 times/30 days) (52-56%). All nutrient intakes analyzed were significantly higher in each of the fish consuming groups as compared to the non-fish consumers ($p<0.05$) with the exception of carbohydrate, saturated fat, alcohol, and vitamin C (Table 2).

Average daily DHA+EPA intake for fish consumers was significantly greater than non-fish consumers ($p<0.00001$). Regular fish consumers' ($\geq$ 9 times/30 days) DHA + EPA intakes were more than double (0.46$\pm$ 0.11 g/day) the DHA+EPA intakes of both the frequent (0.18$\pm$ 0.04 g/day) and infrequent fish consumers (0.07$\pm$ 0.01g/day).

Of the blood lipids tested, HDL concentrations were higher in the fish consumption groups compared to the non fish consumers ($p=0.0011$), while no differences existed across groups for any of the other lipids or CRP (Table 3). The women in this sample had desirable average blood lipid profiles; TC was 188.4 $\pm$ 1.4 mg/dL and HDL 55.7 + 0.7 mg/dL. The

average CRP was 0.36 ± 0.02 mg/L, below the value of 1 mg/L which indicates inflammation (16).

Total blood Hg concentrations increased gradually for each fish consumption group, with fish consumers having significantly greater concentrations than non-fish consumers ($p<0.00001$) (Table 3). Regular fish consumers (≥ 9 times/30 days) had a seven-fold greater total blood Hg concentration than non-fish consumers (4.2 ± 0.7 ug/L vs. 0.6 ± 0.05 ug/L).

Fish Consumption, Lipid Profile and CRP

Initial analyses adjusting for age and energy demonstrated significant negative associations between total 30-day frequency of fish consumption and CRP concentrations (95% CI: -0.19 to -0.02, p=0.015) and significant positive associations with HDL concentrations (95% CI: 0.31 to 2.5, p=0.014). After adjustments for known risk factors for CHD (BMI, educational attainment, ethnicity, self reported physical activity, self reported use of anti inflammatory or lipid lowering prescription drugs, serum cotinine, vitamin C, selenium, fiber, alcohol, mono-unsaturated fatty acids and saturated fatty acids) there were not any changes in the associations between 30-day frequency of fish consumption and CRP, and HDL concentrations (Table 4). Total 30-day frequency of fish consumption was negatively associated with TC in all models ($p>0.05$).

Comment [NRS3]: Start with the most important finds first and leave the non-significant findings to thie end of this subheading. Careful of redundant information.

Sensitivity analyses conducted on the subset of women aged 16-49 years with measured data for TG (n=577) demonstrated that after adjustment for age and other known CHD risk factors, 30-day frequency of fish consumption was negatively associated with TG concentrations (95% CI: -8.0 to -0.33, p=0.034) and calculated LDL concentrations ($p>0.05$) (Table 4).

The calculated 24-hour dietary intake of DHA+EPA was not associated with concentrations of CRP or the blood lipids HDL and TC (data not provided). However, DHA+EPA was negatively associated with LDL concentrations (95% CI: -12.9 to -0.04, p=0.049) after adjustment for age and other known CHD risk factors but DHA+EPA was no longer significantly associated with LDL concentrations after the addition of total blood Hg to the model (95% CI: -13.1 to 0.24, p=0.06).

Fish Consumption, Blood Lipids, CRP and Total Blood Mercury

The association between total blood Hg concentrations (log transformed values) and demographic variables was analyzed using multiple linear regressions (Table 5). Women in the 30-39 years and 40-49 years age categories had significantly greater total blood Hg concentrations than women aged 16-19 years ($p<0.05$). Compared to the other race/ethnicity groups, non-Hispanic blacks had significantly greater total blood Hg concentrations (95% CI: 0.14 to 0.58, p=0.002). After adjusting for age, energy, BMI, education, ethnicity, physical activity, prescription drug use (anti inflammatory or lipid lowering), serum cotinine, vitamin C, selenium, fiber, alcohol, mono-unsaturated fatty acids, and saturated fatty acids, total blood Hg concentrations were significantly higher in all fish consumption groups compared to the non fish eaters, indicating a high correlation between fish intake and total blood Hg concentrations.

The addition of total blood Hg (log) in the Model 3 regression analyses showed attenuation of the association between total 30-day frequency of fish consumption and HDL concentrations to non-significant levels (95% CI: -0.60 to 1.6, p=0.372). To test this attenuation effect we calculated the mean HDL concentration for each group of fish consumers separated by tertile of total blood mercury concentration (Table 6). This analysis showed an increasing trend in HDL cholesterol with increasing tertile of mercury intake demonstrating the high collinearity between fish intake and total blood Hg concentrations.

The addition of total blood Hg (log) in Model 3 for CRP did not cause appreciable changes in the association with CRP concentrations (95% CI: -0.18 -0.03, p=0.010) (Table 4). Similar to the analysis conducted with HDL, the average CRP concentration was calculated by fish consumption group and tertile of total blood mercury concentration. The average CRP concentrations were similar for the different fish frequency consumption groups (data not shown) as well as for fish versus non-fish consumers and increased with each tertile of total blood Hg (Table 7).

Addition of total blood mercury (log) in Model 3 for the relationship between total frequency of fish consumption and TG resulted in a non-significant association between total frequency of fish and TG concentrations (95% CI: -6.0 to 1.44, p=0.183). A follow up analysis to test this affect (i.e calculated average TG by tertile of total blood Hg and fish consumption category) showed a decrease in TG for each tertile of total blood Hg, again demonstrating the high collinearity between fish consumption and total blood Hg concentrations (data not shown).

Discussion

The results of this study suggest an association between frequent fish intake and selected risk markers of CHD (HDL, TG, and CRP) in women aged 16-49 years. As the total 30-day frequency of fish consumption increased, concentrations of HDL increased while concentrations of TG and CRP decreased. The beneficial effects of fish on HDL concentrations were not diminished as total blood Hg concentrations became elevated. The presence of Hg in blood had less impact on CRP levels. Further analyses of the effects of total blood Hg on HDL and CRP by fish consumption group indicated that there was strong collinearity between fish consumption and total blood Hg concentrations, thus total blood Hg does not abate the benefits

of fish consumption. No significant associations were seen between fish intake and either LDL or TC concentrations.

The positive effects of fish intake on HDL concentrations and incidence of CHD have been reported in both men and women (4, 27, 71). The results of this study support previous findings that fish consumption does not consistently affect TC concentrations (4, 7, 72).

Organic (methyl) Hg is present in fish due to its uptake from environmental sources (32). In humans, the sole source of exposure to organic Hg is the consumption of fish and sea mammals (33). Research has linked Hg ingestion from fish to increased risk of CHD in men (2-5, 7). In our study of women aged 16-49 years, total blood Hg seemed to diminish the positive associations between 30-day frequency of fish consumption and HDL concentrations; however further analyses on this effect demonstrated that it was due to collinearity between fish consumption and total blood Hg. Attenuations in risk of CHD due to Hg exposure were reported in studies including men (2-5, 7). However, inconsistent relationships between biomarkers of Hg and CHD risk have been reported, perhaps due to differences in the ages of the study subjects, frequency of fish consumption among populations studied (i.e. Americans eat less fish than Finns), type of fish consumed, and health status of the study samples (6-8).

Our further analysis showed that fish consumers had higher mean HDL concentrations in every tertile of total blood Hg, suggesting that total blood Hg concentrations do not abate the benefits of fish consumption. Total blood Hg concentrations also did not affect the relationship between total 30-day frequency of fish consumption and CRP. It is possible that MeHg does not affect inflammation or that concentrations of Hg in fish are not high enough to significantly affect inflammation. Our further analysis of CRP concentrations by tertile of total blood Hg and

fish consumption group demonstrated the high correlation between fish consumption and total blood Hg, thus CRP concentrations increased with tertile of total blood Hg.

A small study conducted in 10 healthy individuals showed that an induced low-dose Hg exposure caused by the removal of dental amalgams resulted in significant plasma Hg concentrations 24-hours post amalgam removal. However, there was no significant effect of Hg on CRP concentrations up to 7 days post-removal (73). In a follow-up study, 1 g of pulverized amalgam powder was administered to 11 healthy adults. This low dose exposure to MeHg caused significant increases in blood Hg concentrations but did not result in any significant changes in plasma CRP concentrations. MeHg may not affect CHD risk with respect to inflammatory mechanisms such as CRP (74).

CRP is a predictor of CHD risk (11) and is affected by numerous lifestyle variables. In general, individuals with elevated CRP concentrations tend to smoke, have high blood pressure, are overweight, and fail to exercise (13). A few studies have evaluated the effects of dietary intakes of DHA+EPA on CRP concentrations and other biomarkers of inflammation in different samples of subjects and have produced varying results (21, 55). The calculated 24-hour dietary intakes of DHA+EPA in our study were not associated with CRP concentrations.

DHA+EPA have been shown to positively affect HDL and TG concentrations. Men and women supplemented with 2.8 g/day DHA had an 8% increase in HDL, a 28% decrease in TG, and no significant changes in LDL or TC concentrations (55). DHA and EPA may be cardio-protective by lowering plasma concentrations of TG (30, 75). Our sensitivity analysis conducted on 577 women showed protective effects when 30-day frequency of fish consumption was used as the independent variable and TG as the dependent variable. The average DHA+EPA intake in our study (0.1 g/day) was much lower in comparison to that of

other studies. Our results agree with other studies, which have shown that fish intake or markers of fish intake (plasma PUFA) do not significantly impact LDL or TC levels (4, 7). The 24-hour calculated DHA+EPA intake in our study was also less than the AHA recommendation (at least 0.5-1.0 g of omega-3 fatty acids per day to achieve cardio-protective effects) which may explain the lack of association in the models where HDL, LDL, TC were the independent variables (76). When 24-hour dietary intake of DHA+EPA was used as a marker of fish consumption there were no cardio-protective effects in risk markers of CHD. The lack of association is most likely because DHA+EPA intakes were calculated by NCHS based upon 24-hour dietary recall data, from which fish intake is variable. One-day dietary intake records do not represent average long-term consumption especially in foods such as fish that are not commonly consumed.

This cross sectional study extends the results of previous studies using the NHANES dataset (1999-2000). The average total blood Hg (log) was higher for the 1,245 women (aged 16 to 49 years) in the current study than Hg levels reported from the same dataset using a different sample of 1,709 women (1.6 vs. 1.02 ug Hg/L) (38). Schober et al, (2003) also reported that women who ate 3 or more servings of fish (in the past 30 days) had four fold greater geometric mean Hg concentrations compared with non fish consuming women (38). A second study that analyzed hair Hg data reported a three fold higher hair Hg concentrations in frequent fish consumers ($\geq$ 3 times in the past 30-days) than in non-fish consumers (65). In the current study, there was a seven fold difference between women who consumed fish $\geq$ 9 times/30 days and the non-fish consumers. The differences between studies are most likely due to how the fish consumption groups are stratified and the sample sizes within these groups.

When total blood Hg data were analyzed by race/ethnicity, the results of the current study are consistent with two recent reports that analyzed relationships between calculated organic blood Hg (rather than total blood Hg concentrations), hair Hg concentrations, and fish intake during the first two years of the NHANES (1999-2000) (64, 65). The lowest blood organic MeHg concentrations were in Mexican Americans and the highest concentrations were in participants who designated themselves in the "other" racial/ethnic category, which included Asians, Native Americans, and Pacific Islanders (64). Mahaffey reported that women aged 30-49 years has blood organic mercury concentrations 1.5 times greater than women aged 16-29 years. Similarly, we found a two-fold difference in total blood mercury concentrations between women aged 16-19 and 30-39 years (1.0 ± 0.1 vs. 2.0 ± 0.3 ug/L).

Only 57 women in our sample reported consuming fish $\geq$9 times in the past 30 days, a frequency similar to the AHA recommendation of eating fish at least twice a week. Most of the women were either non-fish consumers (n=441) or light fish consumers (1-4 times/30 days, n=166), and thus failed to meet the AHA guideline for fish.

Self-reported exercise may not accurately reflect the participant's true physical activity levels. The fish consumption patterns were approximately the same for each of the 3 physical activity groups analyzed.

Concerns about organic Hg exposure have focused on its negative impacts on the neurodevelopment of infants and young children. Mercury data on other populations and data derived from large surveys are lacking. Our study was limited to a sub-sample of women ages 16 to 49 years in the NHANES (1999-2002) dataset for who total blood Hg data were available. Therefore, these results cannot be generalized to other population groups. Inherent to a large cross sectional study is the temporality of the data therefore, in this observational and cross-

sectional study we cannot exclude the possibility that the cardio-protective effects of fish and DHA+EPA and the effect of total blood Hg on HDL may be due to un-measured variables. However, we included commonly analyzed variables that are available in the NHANES public release data and are known to affect risk of CHD.

Omega-6 and omega-3 PUFAs compete during metabolism and an excessive intake of omega-6 fatty acids may attenuate the cardio-protective benefits normally seen with omega-3 PUFAs. In a large prospective cohort study, Mozaffarian et al (2005) investigated the joint effects of different PUFAs on the risk of CHD in men. These researchers saw benefits in the combination of plant and marine based omega-3 PUFAs on CHD risk independent of omega-6 PUFA consumption. They concluded that non-marine based omega-3 PUFAs are beneficial in reducing risk of CHD, especially when populations do not have easy access or availability to marine sources (77). The interplay of plant derived omega-3 fatty acids were not analyzed in the current study

Finally, the results of this study would have been strengthened if data on LDL and TG were available for more subjects, since both of these lipid biomarkers may be affected by omega-3 fatty acids (33). However, the analysis on a subpopulation did not show any association between omega-3-fatty acids and these blood lipids. Other inflammatory biomarkers such as Interleukin-6, or other cytokines may be better indicators of CHD risk than CRP, but these data were not available in the current NHANES surveys.

The current study included two measures of fish intake, a 30-day frequency of fish intake and a second 24-hour dietary recall interview that assessed DHA+EPA intake. The 30-day fish FFQ provides a good estimate of habitual dietary patterns that may reveal the potential benefits of fish consumption. This instrument also underscores the pitfall of using 24-hour

dietary recall data for a food that is not regularly consumed by the study subjects. Another strength is that the current study included healthy women of childbearing age, whereas studies that examined the joint association between fish intake, methyl mercury and CHD only included men and older women. Finally, because the NHANES dataset is large, we were able to control for a number of demographic and lifestyle variables.

Summary

The results of this study provide further support for recommending regular fish consumption along with maintaining a healthy lifestyle (e.g., not smoking and getting daily moderate exercise) as a way of reducing the risk of CHD. The effect of mercury on biomarkers of inflammation is unclear. Based on this study and others, it appears that the levels of mercury in fish are not high enough to significantly impact the risk of CHD in healthy women of childbearing age. Furthermore, the levels of omega-3 fatty acids in fish may be adequate to offset the risks of ingesting mercury in fish. Future studies using the NHANES (1999-2000) 30-day fish food frequency questionnaire should separately analyze the relationships between fatty fish intake and total fish intake on health-related variables. Further research is also needed to investigate interactions between diet, mercury, and health in women of all ages.

Tables

Table 1. Characteristics of the study sample by fish consumption group

Characteristic	Non Fish Consumers (n=441)	Fish Consumers 1 to 4 times / 30 days (n=616)	Fish Consumers 5 to 8 times / 30 days (n= 131)	Fish Consumers ≥ 9 times/ 30 days (n=57)	P[1]
Age (y)	28.9 ± 0.6	33.5 ± 0.5	35.4 ± 1.2	33.9 ± 1.5	<0.00001
BMI (kg/m^2)	26.0 ± 0.6	26.6 ± 0.3	25.8 ± 0.6	26.9 ± 0.9	NS
Ethnicity (%)					
Mexican American	43.2	48.0	6.4	2.4	
Non-Hispanic white	26.7	54.0	14.2	5.1	
Non-Hispanic black	24.4	51.8	16.3	7.5	
Other Hispanic and Other	26.2	53.0	10.5	10.3	
Serum cotinine (ng/mL)	46.1 ± 6.8	46.8 ± 6.8	39.7 ± 6.7	15.0 ± 6.2	0.0043
Physical activity readiness code (%)					
Little or no exercise [1,2]	29.4	54.5	11.3	4.8	
Moderate exercise [3,4]	26.3	52.0	15.4	6.3	
Heavy exercise [5-8]	28.1	55.7	10.3	5.9	

Summaries presented as Mean ± SEM for the continuous variables and percentage in each category for the categorical variables

[1] Adjusted Wald Test to test the accumulated difference of all fish consumers versus non- fish consumers

[2] Little or no regular recreation, sport or physical activity and avoids walking or exertion

[3] Little or no regular recreation, sport or physical activity but walks for pleasure and occasionally exercise

[4] Participating regularly in recreation or work requiring modes physical activity for 10 to 60 minutes per week.

[5] Participating regularly in recreation or work requiring modest physical activity for more than 60 minutes per week.

[6] Participating regularly in heavy physical activity for less than 30 minutes per week.

[7] Participating regularly in heavy physical activity for 30-60 minutes per week.

[8] Participating regularly in heavy physical activity for 1-3 hours per week.

[9] Participating regularly in heavy physical activity for more than 3 hours per week.

Table 2. Nutrient intake profile of the study sample by fish consumption group (Mean ± SEM)

Nutrient[1]	Non Fish Consumers (n=441)	Fish Consumers 1 to 4 times / 30 days (n=616)	Fish Consumers 5 to 8 times / 30 days (n=131)	Fish Consumers ≥ 9 times/ 30 days (n=57)	P [2]
Kcals (kcal/day)	1918 ± 42.2	1975 ± 36.1	2110 ± 78.3	2252 ± 122.1	0.028
Protein (g/day)	65.1 ± 2.0	68.6 ± 1.4	79.3 ± 4.3	86.7 ± 4.4	0.0003
Carbohydrate (g/day)	254.5 ± 6.0	251.7 ± 5.6	266.1 ± 9.1	267.7 ± 20.4	NS[23]
Fiber (g/day)	12.9 ± 0.5	13.9 ± 0.5	14.6 ± 0.8	16.1 ± 1.2	0.0451
Fat (g/day)	69.5 ± 2.3	74.9 ± 1.6	78.8 ± 4.6	89.2 ± 5.3	0.020
Saturated fatty acids (g/day)	23.3 ± 0.9	24.8 ± 0.6	25.4 ± 1.4	27.8 ± 2.3	NS
Monounsaturated fatty acids (g/day)	26.3 ± 0.95	28.0 ± 0.6	28.9 ± 1.8	33.5 ± 1.9	0.042
Polyunsaturated fatty acids (g/day)	14.0 ± 0.6	15.4 ± 0.5	16.7 ± 1.1	19.7 ± 1.6	0.007
Alcohol intake (g/day)	6.0 ± 1.2	7.6 ± 1.2	7.9 ± 1.5	10.6 ± 3.8	NS
Cholesterol (g/day)	217.9 ± 12.0	223.6 ± 8.4	280.4 ± 21.9	299.8 ± 24.7	0.042
Vitamin C (mg/day)	83.7 ± 7.1	79.1 ± 3.9	92.9 ± 7.8	104.1 ± 18.1	NS
Selenium (mcg/day)	83.8 ± 2.9	91.6 ± 2.4	108 ± 6.1	121.3 ± 8.8	0.0002
DHA + EPA (g/day)	0.03 ± 0.01	0.07 ± 0.01	0.18 ± 0.04	0.46 ± 0.11	<0.00001

[1] Nutrients calculated by NHANES from the 24-hour dietary recall interview

[2] Adjusted Wald Test to test the accumulated difference of all fish consumers versus non- fish consumers

[3] NS indicates p>0.05

Table 3. Blood lipids, C-reactive protein and total blood mercury concentrations by fish consumption group (Mean ± SEM)

Variables of Interest	Non Fish Consumers (n=441)[1]	Fish Consumers 1 to 4 times / 30 days (n=616)[2]	Fish Consumers 5 to 8 times / 30 days (n=131)[3]	Fish Consumers ≥ 9 times/ 30 days (n=57)[4]	P [5]
Total cholesterol (mg/dL)	184.9 ± 2.6	190.6 ± 1.4	185.5 ± 4.5	191.9 ± 3.4	NS[6]
HDL cholesterol (mg/dL)	52.2 ± 1.2	57.1 ± 0.9	55.6 ± 1.2	59.5 ± 2.0	0.0011
LDL cholesterol (mg/dL)	111.4 ± 3.7	109.6 ± 1.9	108.9 ± 5.2	114.6 ± 3.9	NS
Triacylglycerol (mg/dL)	101.3 ± 5.0	104.2 ± 3.5	109.5 ± 13.7	95.8 ± 10.4	NS
CRP (mg/L)	0.35 ± 0.03	0.39 ± 0.03	0.27 ± 0.04	0.30 ± 0.08	NS
Total blood mercury (ug/L)	0.6 ± 0.05	1.6 ± 0.1	2.2 ± 0.3	4.2 ± 0.7	<0.00001

[1] n=195 for LDL, and n=213 for Triacylglycerol
[2] n=288 for LDL, and n=304 for Triacylglycerol
[3] n=64 for LDL, and n=67 for Triacylglycerol
[4] n=30 for LDL, and n=32 for Triacylglycerol
[5] Adjusted Wald Test to test the accumulated difference of all fish consumers versus non- fish consumers
[6] NS indicates p>0.05

Table 4. Summaries of regressions of CHD risk factors on 30-day fish frequency consumption (g/person/day), women aged 16 to 49 years NHANES 1999-2002

Dependent Variable	Sample Size	Model R^2	Square root of fish frequency b (95% CI)	P
C Reactive protein				
Model 1 [1]	1245	0.04	-0.1 (-0.19 -0.02)	0.015
Model 2 [2]	1245	0.33	-0.09 (-0.15 -0.02)	0.011
Model 3 [3]	1245	0.33	-0.10 (-0.18 -0.03)	0.010
HDL cholesterol				
Model 1 [1]	1245	0.04	1.4 (0.31 2.5)	0.014
Model 2 [2]	1245	0.25	1.0 (0.07 2.0)	0.036
Model 3 [3]	1245	0.25	0.48 (-0.60 1.6)	NS
Total cholesterol				
Model 1 [1]	1245	0.12	-1.4 (-3.2 0.36)	NS
Model 2 [2]	1245	0.15	-0.80 (-2.7 1.1)	NS
Model 3 [3]	1245	0.15	-1.7 (-4.5 1.1)	NS
LDL cholesterol				
Model 1 [1]	577	0.07	-2.1 (-5.1 0.84)	NS
Model 2 [2]	577	0.13	-1.8 (-4.8 1.2)	NS
Model 3 [3]	577	0.13	-2.1 (-5.3 1.1)	NS
Triacylglycerol				
Model 1 [1]	577	0.02	-4.4 (-8.7 -0.02)	0.049
Model 2 [2]	577	0.14	-4.2 (-8.0 -0.33)	0.034
Model 3 [3]	577	0.14	-2.3 (-5.9 1.2)	NS

[1] Adjusted for age and energy

[2] Adjusted for Model 1 variables, BMI, education, ethnicity, physical activity, prescription drug use (anti inflammatory or lipid lowering), serum cotinine, vitamin C, selenium, fiber, alcohol, mono-unsaturated fatty acids, saturated fatty acids

3 Adjusted for model 2, log total blood mercury (log), and the interaction term of total blood mercury (log)*square root of fish frequency

Table 5. Regression model for y=log (total blood Hg)[1], Women aged 16 to 49 years

Characteristics	b	(95% CI)	Total Blood mercury (ug/L) (Mean ± SEM)	p
Age Group				
16 to 19 years	-		0.91 ± 0.1	
20 to 29 years	0.22	(-0.05 to 0.48)	1.3 ± 0.1	0.103
30 to 39 years	0.48	(0.22 to 0.73)	1.8 ± 0.2	0.001
40 to 49 years	0.65	(0.34 to 0.96)	1.8 ± 0.2	<0.0001
Race/Ethnicity				
Non-Hispanic white	-		1.5 ± 0.1	
Non-Hispanic black	0.36	(0.14 to 0.58)	1.7 ± 0.2	0.002
Mexican American	0.10	(-0.06 to 0.26)	1.2 ± 0.1	0.201
Other Hispanic and other	0.31	(-0.03 to 0.65)	2.0 ± 0.4	0.075
Fish consumption frequency in past 30 days				
0	-		0.62 ± 0.05	
1 to 4 times	0.83	(0.63 to 1.03)	1.6 ± 0.1	<0.0001
5 to 8 times	1.17	(0.89 to 1.45)	2.2 ± 0.3	<0.0001
9 + times	1.76	(1.41 to 2.1)	4.2 ± 0.7	<0.0001

[1] Adjusted for age, energy, BMI, education, ethnicity, physical activity, prescription drug use (anti inflammatory or lipid lowering), serum cotinine, vitamin C, selenium, fiber, alcohol, mono-unsaturated fatty acids, saturated fatty acids

Table 6. Average HDL concentration by total blood Hg tertile and fish versus non fish consumers (mg/L), women aged 16 to 49 years NHANES 1999-2002

Fish Group		Total Blood Hg Tertile			
		1	2	3	Total
Non fish consumers	Mean	51.5	54.1	50.4	52.2
	95% CI	48.8 to 54.1	48.9 to 59.3	48.0 to 52.7	
Fish consumers	Mean	52.5	56.6	59.6	57.0
	95% CI	50.0 to 55.1	54.6 to 58.6	57.2 to 62.0	
Total	Mean	49.3	56.0	58.9	

Table 7. Average CRP concentration by total blood Hg tertile and fish versus non fish consumers (mg/dL), women aged 16 to 49 years NHANES 1999-2002

Fish Group		Total Blood Hg Tertile			
		1	2	3	Total
Non fish consumers	Mean	0.14	0.17	0.14	0.15
	95% CI	0.11 to 0.17	0.14 to 0.2	0.07 to 0.25	
Fish consumers	Mean	0.17	0.13	0.15	0.15
	95% CI	0.14 to 0.2	0.11 to 0.15	0.12 to 0.18	
Total	Mean	0.18	0.22	0.45	

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Chapter 4: Implications and Future Research

Health implications

Fish intake of at least two servings per week is beneficial for maintaining desirable blood lipid profiles and markers of inflammation and was also associated with an overall healthier lifestyle (lower exposure to smoking, lowest number of non-exercisers, greatest intake of various nutrients known to be beneficial such as fiber, mono-unsaturated fat, DHA+EPA, vitamin C and selenium). Our research showed that people in the two highest fish consuming categories had the lowest CRP concentrations compared to non-fish and light fish consumers despite intervention data not showing significant differences. Our findings agree with other published observational data and provide further support for the AHA fish consumption recommendations.

In addition to supporting the AHA fish recommendations, the results of this observational study are consistent with other observational research findings on the cardiovascular benefits of fish consumption, specifically on CHD risk markers. Despite the contamination of many varieties of fish, the limited sample of female fish consumers of childbearing age in the US had desirable blood lipid profiles. The current research shows that despite both the risks and benefits of fish consumption, moderate fish intakes will result in increased HDL and reduced TG concentrations, thus may reduce the risk of CHD. People interested in these effects may begin to incorporate fatty fish known to have lower or limited mercury concentrations into a balanced diet; however maintain awareness of fish known to have greater mercury concentrations than others.

Consumers in the US are often provided with cautionary statements regarding fish and the contaminants often found in fish, including PCBs and mercury. The current research my be included with future observational and intervention studies which may find that the mercury concentrations in fish do not outweigh the benefits of fish consumption, resulting in reduced fear of fish consumption by the US population and increased fish intakes.

Importance of the research

The results of our study imply that the concentrations of Hg in blood are not yet great enough to abate the cardio-protective benefits of fish consumption in women aged 16-49 years; however, additional research on high CHD risk populations may show different results. Starting in 2002, the NHANES began collecting blood mercury data on the sample population aged $\geq$1 year. These measurements will provide a reference point for the women of childbearing age from which results of further studies on the joint effects of DHA+EPA and mercury can be compared. Our study provides a basis for preliminary comparison for these future studies.

Additional research is necessary to understand if there exists a threshold concentration where the risks of certain fish outweigh the benefits. The lack of strong association between Hg and the CHD risk markers may imply that the concentrations of Hg in our food supply are not great enough to cause concern for cardiovascular health and that consumers at greater risk for CHD are better off eating fish for the beneficial properties (high protein, low fat, rich in antioxidants and omega-3 fatty acids) rather than selecting foods that may not have these beneficial attributes. On the other hand, the weak association between Hg and the CHD risk markers may have been attributed to the younger age of our population group which is not typically at high risk of CHD. Our study adds to the growing body of observational data

demonstrating the benefits of fish consumption; despite the mixed findings in intervention studies. Additional clinical and intervention research is necessary to better understand the role of fish consumption, omega-3 fatty acid intake and Hg intake on CHD risk.

Suggestions for further research

Three studies have been published using data from the first two years of the NHANES data (38, 64, 65). Ideas for future research would be to replicate these studies using the 1999-2002 dataset. In addition the NHANES 1999-2002 data could be used to determine how many women have total blood Hg concentrations greater than 5.8 ug/L and if these women with >5.8 ug/L total blood Hg also have high reported fish consumption? If not, researchers should review other NHANES data (food intake records, dental history) for the possible reason for these elevated Hg concentrations.

In addition to a high total blood Hg analysis, further studies could be conducted to investigate any possible relationships between specific varieties of fish and the overall health profiles of consumers (especially dark fatty fish known to be excellent sources of omega-3 fatty acids as well as fish known to be high in mercury). Examples of research questions to investigate include: (1) Do significant differences exist between the risk markers of CHD in non-tuna consumers, light tuna consumers and heavy tuna consumers? and (2) do populations at the highest risk for CHD not report consuming fish?

Our research also showed that the group composed of other Hispanic and other ethnicities had the greatest measured total blood mercury concentrations. A future evaluation of the diet, lipid biomarkers and CHD risk markers of this population group may provide more insight into the risk benefit paradigm in consuming fish. In addition, the dietary patterns

associated with fish consumption in this race/ethnicity group may reveal healthy food consumption habits also associated with decreased CHD risk.

The lack of findings in the DHA+EPA regression model speaks to the need for longer-term dietary intake data. Precision in estimating dietary intake data is necessary for policy makers and public health professionals to best understand what the US population is consuming. Future releases of NHANES data will incorporate a second day of dietary data, which is essential to capture the intakes of foods not commonly consumed, such as fish. When researchers, public health and policy professionals use nationally representative food intake data to estimate exposure to foods, nutrients, or contaminants in foods or nutrients, from foods that are not regularly consumed (e.g. fish), the result will underestimate the actual population exposure and may result in unnecessary cautionary statements due to lack of better data.

Even for those consumers who reported fish twice weekly, mean DHA+EPA intakes in our study were still below the AHA 1 g/day recommendations (0.46 ± 0.11 g/day). This may indicate that the women who reported eating fish did not eat dark fatty fish or other fish known to contain the healthy omega-3 fatty acids. Clinical studies have failed to show significant associations between omega-3 fatty acids (both plant and marine based) and inflammatory markers (78-80); however, observational studies have shown otherwise (25, 81). Further research is needed to better understand the anti-inflammatory effects of fish consumption and if any effects are due to omega-3 fatty acids or a combination of nutrients found in fish or dietary patterns associated with fish intake, not just omega-3 fatty acids.

There exists research on the effects of consumer awareness of state and local sport-fish advisories and fish consumption. It is possible that the currently available consumer advisories are deterring the general public from consuming fish. Despite the current advisory being very

specific to fish type, the public that is already bombarded with conflicting nutritional information may be generalizing the health concerns of Hg and other chemically contaminated fish varieties to all fish. Continued consumer awareness studies need to be conducted with the arrival of new observational and clinical data regarding the risks and benefits of fish intake.

Appendix 1: List of lipid altering drug ingredients

Lipid Altering Standard Generic Ingredient Name	FDA Drug Class Code
ALUMINUM CHLORIDE	411
AMILORIDE HYDROCHLORIDE; HYDROCHLOROTHIAZIDE	506
AMLODIPINE BESYLATE	3570
AMLODIPINE BESYLATE; BENAZEPRIL HYDROCHLORIDE	510
ATENOLOL	4024
ATENOLOL; CHLORTHALIDONE	506
ATORVASTATIN CALCIUM	7296
BENAZEPRIL HYDROCHLORIDE	2024
BISOPROLOL FUMARATE; HYDROCHLOROTHIAZIDE	2530
CANDESARTAN CILEXETIL	1012
CAPTOPRIL	1012
CARVEDILOL	506
CLONIDINE	1518
CLONIDINE HYDROCHLORIDE	506
DIGOXIN	501
DILTIAZEM HYDROCHLORIDE	1004
ENALAPRIL MALEATE	1518
FLUVASTATIN SODIUM	506
FOSINOPRIL SODIUM	1518
FUROSEMIDE	2024
GEMFIBROZIL	1008
HYDROCHLOROTHIAZIDE	6072
HYDROCHLOROTHIAZIDE; IRBESARTAN	506
HYDROCHLOROTHIAZIDE; LISINOPRIL	1012
HYDROCHLOROTHIAZIDE; TRIAMTERENE	5566
HYDROCHLOROTHIAZIDE; VALSARTAN	506
HYDROXYZINE HYDROCHLORIDE	506
IRBESARTAN	506
LABETALOL HYDROCHLORIDE	1012
LISINOPRIL	5060
METOPROLOL SUCCINATE	503
METOPROLOL TARTRATE	503
NADOLOL	503
NIFEDIPINE	503
PROPRANOLOL HYDROCHLORIDE	1506
QUINAPRIL HYDROCHLORIDE	1028
RAMIPRIL	1012
SIMVASTATIN	912
TERBUTALINE SULFATE	1024
VALSARTAN	506
WARFARIN SODIUM	409

Appendix 2: List of anti-inflammatory drug ingredients

Inflammatory Altering Standard Generic Ingredient Name	FDA Drug Class Code
ACETAMINOPHEN	3440
ACETAMINOPHEN; CODEINE PHOSPHATE	36120
ACETAMINOPHEN; DICHLORALANTIPYRINE; ISOMETHEPTENE MUCATE (1:1)	7016
ACETAMINOPHEN; HYDROCODONE BITARTRATE	55040
ACETAMINOPHEN; OXYCODONE HYDROCHLORIDE	30960
ACETAMINOPHEN; PENTAZOCINE HYDROCHLORIDE	1720
ACETAMINOPHEN; PROPOXYPHENE NAPSYLATE	27520
ACRIVASTINE; PSEUDOEPHEDRINE HYDROCHLORIDE	1945
ACYCLOVIR	1074
ADAPALENE	3795
ALBUTEROL	133860
ALBUTEROL SULFATE; IPRATROPIUM BROMIDE	3880
AMMONIUM LACTATE	1265
AMOXICILLIN	15224
AMOXICILLIN TRIHYDRATE; CLAVULANATE POTASSIUM	2422
AMPICILLIN	1038
ASPIRIN	3440
ASPIRIN; BUTALBITAL; CAFFEINE	1044
ASPIRIN; OXYCODONE HYDROCHLORIDE; OXYCODONE TEREPHTHALATE	3440
ATROPINE SULFATE; DIPHENOXYLATE HYDROCHLORIDE	875
AZATHIOPRINE	1181
AZELASTINE HYDROCHLORIDE	3888
AZITHROMYCIN DIHYDRATE	2088
BENZOYL PEROXIDE	1265
BENZOYL PEROXIDE; ERYTHROMYCIN	1265
BIRTH CONTROL DRUG-UNSPECIFIED	3120
BUTABARBITAL; HYOSCYAMINE HYDROBROMIDE; PHENAZOPYRIDINE HYDROCHLORIDE	354
CARISOPRODOL	5492
CEFACLOR	1041
CEFADROXIL	347
CEFUROXIME AXETIL	1388
CELECOXIB	18964
CEPHALEXIN	5205
CETIRIZINE HYDROCHLORIDE	31104
CHLORPHENIRAMINE MALEATE	1944
CHLORPHENIRAMINE POLISTIREX; HYDROCODONE POLISTIREX	1945
CIMETIDINE	874
CIPROFLOXACIN HYDROCHLORIDE	3550
CLARITHROMYCIN	3828
CLINDAMYCIN HYDROCHLORIDE	1044

Inflammatory Altering Standard Generic Ingredient Name	FDA Drug Class Code
CLINDAMYCIN PHOSPHATE	696
CODEINE PHOSPHATE	1720
CYCLOBENZAPRINE HYDROCHLORIDE	9611
CYCLOSPORINE	347
DESLORATADINE	3888
DESOGESTREL; ETHINYL ESTRADIOL	16544
DEXTROMETHORPHAN HYDROBROMIDE; GUAIFENESIN; PSEUDOEPHEDRINE HYDROCHLORIDE	1940
DICLOFENAC SODIUM	4704
DICLOXACILLIN SODIUM	346
DICYCLOMINE HYDROCHLORIDE	877
DIOXYBENZONE; HYDROQUINONE; PADIMATE O	1265
DIPHENHYDRAMINE HYDROCHLORIDE	2742
DIRITHROMYCIN	348
DOXYCYCLINE	2800
DOXYCYCLINE HYCLATE	350
ERYTHROMYCIN	2429
ESTRADIOL	10340
ESTRADIOL; NORETHINDRONE ACETATE	1034
ESTROGENS, CONJUGATED SYNTHETIC A	1034
ESTROGENS, CONJUGATED; MEDROXYPROGESTERONE ACETATE	5170
ESTROGENS, ESTERIFIED	2068
ESTROPIPATE	2068
ETANERCEPT	1724
ETHINYL ESTRADIOL; ETHYNODIOL DIACETATE	6204
ETHINYL ESTRADIOL; LEVONORGESTREL	35156
ETHINYL ESTRADIOL; NORETHINDRONE	25850
ETHINYL ESTRADIOL; NORETHINDRONE ACETATE	14560
ETHINYL ESTRADIOL; NORGESTIMATE	66176
ETHINYL ESTRADIOL; NORGESTREL	9306
ETODOLAC	1720
FAMCICLOVIR	776
FAMOTIDINE	1748
FEXOFENADINE HYDROCHLORIDE	25272
FEXOFENADINE HYDROCHLORIDE; PSEUDOEPHEDRINE HYDROCHLORIDE	9725
FLUCONAZOLE	716
FLUTICASONE PROPIONATE	8855
FLUTICASONE PROPIONATE; SALMETEROL XINAFOATE	1940
GRISEOFULVIN, MICROCRYSTALLINE	358
GUAIFENESIN	1940
GUAIFENESIN; PSEUDOEPHEDRINE HYDROCHLORIDE	5820
HYDROCORTISONE	877
HYDROXYCHLOROQUINE SULFATE	1724
IBUPROFEN	55040
INTERFERON BETA-1A	388
IPRATROPIUM BROMIDE	1940

Inflammatory Altering Standard Generic Ingredient Name	FDA Drug Class Code
ISONIAZID	712
ITRACONAZOLE	358
KETOCONAZOLE	716
KETOPROFEN	1720
KETOROLAC TROMETHAMINE	3136
LANSOPRAZOLE	5244
LEVONORGESTREL	1037
LEVOTHYROXINE SODIUM	36295
LOPERAMIDE HYDROCHLORIDE	875
LORATADINE	23280
MEDROXYPROGESTERONE ACETATE	7238
MESTRANOL; NORETHINDRONE	4136
METRONIDAZOLE	1775
MINOCYCLINE HYDROCHLORIDE	1400
MOMETASONE FUROATE MONOHYDRATE	1947
MOXIFLOXACIN HYDROCHLORIDE	357
NAPROXEN	15480
NAPROXEN SODIUM	3440
NITROFURANTOIN MONOHYDRATE; NITROFURANTOIN, MACROCRYSTALLINE	354
NIZATIDINE	874
NORETHINDRONE	2068
NORETHINDRONE ACETATE	1034
OFLOXACIN	355
OMEPRAZOLE	3496
OXAPROZIN	1722
PENICILLIN G PROCAINE	346
PENICILLIN V POTASSIUM	692
PENICILLIN-UNSPECIFIED	692
PHENAZOPYRIDINE HYDROCHLORIDE	708
PROGESTERONE	2068
PROPYLTHIOURACIL	1037
RABEPRAZOLE SODIUM	874
RANITIDINE HYDROCHLORIDE	3496
RIZATRIPTAN BENZOATE	1723
ROFECOXIB	10362
SALMETEROL XINAFOATE	1940
SULFAMETHOXAZOLE; TRIMETHOPRIM	1050
SUMATRIPTAN SUCCINATE	3446
THYROID	3111
TRETINOIN	6325
VALACYCLOVIR HYDROCHLORIDE	776
ZAFIRLUKAST	1940

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