Novel Studies of Fat and Nutrient Intakes and the Risk of Human Cancers

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Dedication

This dissertation is dedicated to the individuals I have known and loved who have suffered from cancer: my grandfathers Clarence “Mike” Ainslie and Lionel Lewis, my cousin Elizabeth Ainslie-Lynch, and my loving cat Boo Boo.

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List of Abbreviations

%CV	% Coefficient of variation
13-HODE	13-Hydroxy-octadecadienoic acid
16α-OHE_1	16α-Hydroxyestrone
2-OHE_1	2-Hydroxyestrone
4-OHE	4-Oxo-2-*trans*-hexenal
6β-OHC	6β-Hydroxycortisol
8-OHdG	8-Hydroxy-2'-deoxyguanosine
8-oxoGua	8-Oxo-7,8-dihydroguanine
95% CI	95% Confidence interval
9-HODE	9-Hydroxy-octadecadienoic acid
AA	Arachidonic acid
ACC	Acetyl CoA-carboxylase
ADH	Alcohol dehydrogenase
AKR	Aldo-keto reductase
ALA	α-linolenic acid
ALDH	Aldehyde dehydrogenase
ATM	Ataxia telangiectasia
CIN	Cervical intraepithelial neoplasia
COX-2	Cyclooxygenase-2
CpG	Cytosine- and guanine-rich segments of DNA
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DHN	1,4-Dihydroxy-2-*trans*-nonene
DIM	3,3'-Diindolylmethane
DMBA	7,12-Dimethylbenz[a]anthracene
DNPH	2,4-Dinitrophenylhydrazine
EPA	Eicosapentaenoic acid
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
FMO3	Flavin monooxygenase-3
GLA	Gamma linolenic acid
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-*S*-transferase
HDE	4-Hydroxy-2-*trans*-decenal
HHE	4-Hydroxy-2-*trans*-hexenal

HI-IM	1-(3-Hydroxymethyl)indolyl-3-indolylmethane
HNA	4-Hydroxy-2-*trans*-nonenoic acid
HNE	4-Hydroxy-2-*trans*-nonenal
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HR	Hazard ratio
I3A	Indole-3-carboxaldehyde
I3C	Indole-3-carbinol
I3CA	Indole-3-carboxylic acid
ICZ	Indolo[3,2b]carbazole
iNOS	Inducible nitric oxide synthase
IRR	Incidence rate ratio
LA	Linoleic acid
LC/ESI-MS/MS-SRM	Liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring
LDL	Low-density lipoprotein
LOQ	Limit of quantitation
$LTr_{1'}$	2-(Indol-3-ylmethyl)-indol-3-yl]indol-3ylmethane
MDA	Malondialdehyde
MMP	Matrix metalloproteases
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
n-3	Omega-3 fatty acids
NAT-2	*N*-Acetyltransferase-2
NDMA	*N*-Nitrosodimethylamine
NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc	4-(Methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl-b-*O*-D-glucosiduronic acid
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NQO1	NAD(P)H:Quinine oxidoreductase 1
OR	Odds ratio
PEITC	Phenethyl isothiocyanate
PMR	Proportional mortality rate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RR	Relative risk
SFN	Sulforaphane
SIR	Standardized incidence ratio

SMR	Standardized mortality ratio
SOD	Superoxide dismutase
SULT	Sulfotransferase
TBARS	Thiobarbituric acid reactive substances
TLC	Thin layer chromatography
UGT	UDP-glucuronosyltransferase
WHEL	Women's Healthy Eating Living
WINS	Women's Intervention Nutrition Study

Foreword

The following work represents the experiments I completed during my time in the Nutrition Graduate Program at the University of Minnesota. Throughout my time in the program, I was fortunate to gain experience in several laboratories and under the guidance of multiple mentors. My dissertation projects share the common thrust of furthering the knowledge of how nutrition relates to cancer prevention, development, and progression. The projects described herein incorporate my coursework in nutrition, epidemiology, immunology, and cancer biology to advance the understanding of polyunsaturated fatty acids, dietary bioactives, and antioxidants in relation to cancer development and progression while simultaneously establishing methods and data from which to structure future human research.

In the first section, I will review the scientific literature regarding consumption of polyunsaturated fatty acids—specifically omega-3 fatty acids—in regards to breast cancer prevention and treatment. The experiment I conducted to measure the effects of omega-3 fatty acids on *in vivo* lipid peroxidation was part of a crossover feeding study specifically addressing breast cancer risk biomarkers. Although I did not include any of these biomarkers in my analyses, my results are informative and pertain to future research exploring lipid peroxidation and breast cancer risk.

In the subsequent section, I will detail the development of a highly sensitive technique to quantify the exposure of individuals to indole-3-carbinol, a chemopreventive

bioactive compound produced following the consumption of cruciferous vegetables such as Brussels sprouts. This work represents a majority of my experience as a trainee under the Division of Epidemiology's Nutrition and Cancer training grant. The series of validation experiments improved my knowledge of analytical chemistry and biomarker development and measurement immensely, and the resulting technique will prove useful for epidemiological and experimental research of cancer chemoprevention by indole-3-carbinol and its metabolite, 3,3′-diindolylmethane.

The final section of my dissertation addresses statistical analyses to explore the relationships between dietary fish, fat, and antioxidants and the risk of gastric cancer in the prospective cohort of the Singapore Chinese Health Study. This experience allowed me to directly apply the proficiency in epidemiological methods gleaned during my required coursework for the Nutrition and Cancer training grant. Additionally, the work augmented the collaborative skills necessary for population-based research into nutrition and cancer while offering additional information about dietary prevention of this deadly cancer.

These three sections share several commonalities in addition to their broad exploration of nutrition and cancer. Lipid peroxidation metabolites and 3,3′-diindolylmethane function similarly to prevent carcinogenesis, including by up-regulating cancer cell death. Due to their corresponding chemical properties, they follow overlapping metabolic pathways within the body. Another common theme is inflammation, which is associated directly with consumption of polyunsaturated fatty acids as well as cancer development in breast and gastric tissues. Infection of the

stomach with *Helicobacter pylori*, a strong risk factor for non-cardia gastric cancer, increases inflammation while triggering an immune response that may be enhanced or attenuated by dietary constituents including salt, antioxidant vitamins, and omega-3 fatty acids.

Part I: The effect of omega-3 (n-3) fatty acids on *in vivo* lipid peroxidation

Introduction

Consumption of omega-3 fatty acids (n-3) is recommended to promote cardiovascular health, and there is some evidence that n-3 may reduce the risk of cancers, including breast cancer. However, polyunsaturated fatty acids (PUFA) such as n-3 are highly unsaturated and therefore susceptible to lipid peroxidation, an autocatalytic process triggered by the presence of free radicals that produces several reactive metabolites that have been shown to bind DNA and modify proteins. Nevertheless, cell culture and animal studies largely support a protective effect of dietary n-3 on breast cancer risk, at least partially due to lipid peroxidation. Human research has shown lipid peroxidation is increased in breast cancer patients compared to healthy controls but does not support a causative role of lipid peroxidation of n-3 in the development of breast cancer. More research will be needed to characterize the interaction between n-3 and lipid peroxidation in breast carcinogenesis.

Chapter I: Literature Review

Health benefits of n-3 fatty acids

Omega-3 fatty acids (n-3) are polyunsaturated fatty acids (PUFA) classified by the position of their first double-bond at the third carbon from the methyl end of the hydrocarbon chain (Figure 1)[1]. N-3 include the essential fatty acid α-linolenic acid (ALA, 18:3) as well as long-chain n-3, such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6)[2]. Some food sources of n-3 include canola oil, soybean oil, flax seeds, and walnuts, providing ALA, and salmon or cod liver oil, providing EPA and DHA[1].

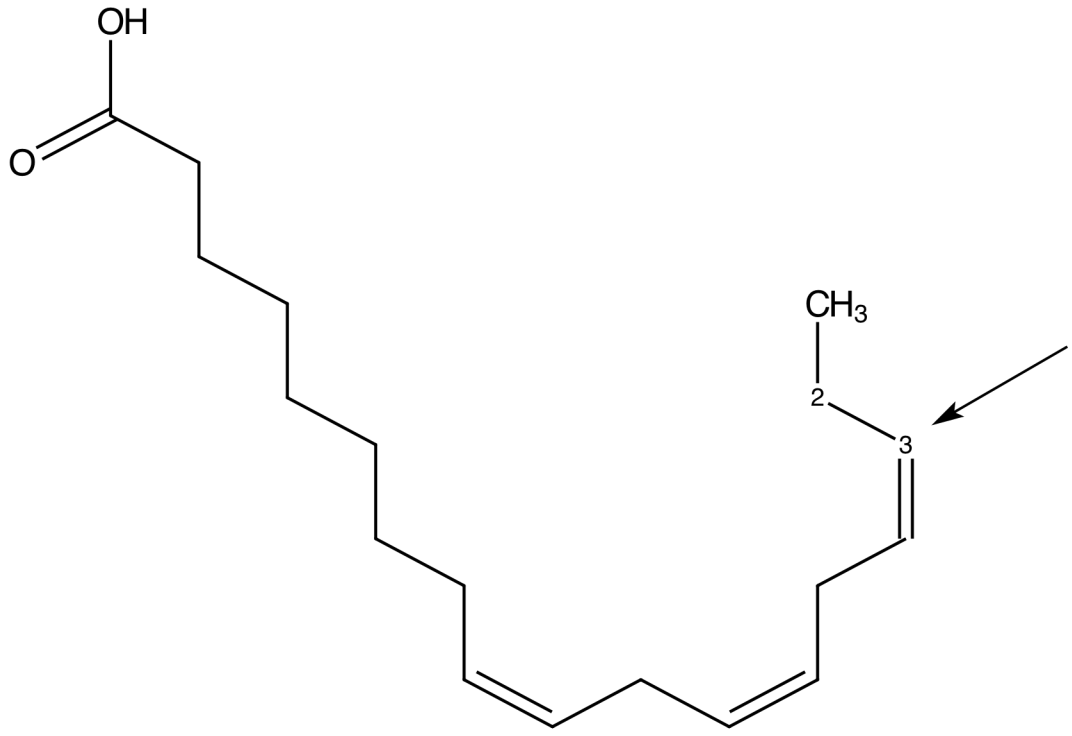


Figure 1. α-Linolenic acid (ALA, 18:3) with double bond denoting omega-3 (n-3) fatty acid classification indicated (adapted from [3]).

Over the past several decades, evidence has accumulated that suggests that increased intakes of n-3, particularly EPA and DHA, are associated with a decreased risk of cardiovascular disease, including sudden cardiac death, cardiac arrhythmia, atherosclerosis, hypertriglyceridemia, and cerebrovascular accident[2, 4]. Although the Institute of Medicine has yet to establish Dietary Reference Intakes for EPA and DHA, the American Heart Association recommends consumption of fatty fish at least twice per week for all adults and consumption of one gram per day of EPA and DHA from fish and/or supplements in individuals with coronary heart disease[5].

Chemopreventive effects of n-3 fatty acids

More recently, there has been increasing interest in the role of n-3 in the prevention of several human cancers. In a seminal review, Terry et al.[6] concluded that while there was some evidence that high intakes of fatty fish or n-3 reduce the risk of breast cancer, particularly among Japanese and Norwegian women, many epidemiological studies yielded null results, indicating no significant effect of such dietary exposures on breast cancer risk. A subsequent review by MacLean et al.[7] reached similar conclusions. The benefit of both reviews is the inclusion of results from large data sets among free-living populations, which provide more generalizable results. However, such studies are subject to the limitations of all epidemiological studies including measurement error, dietary variability during the years of follow-up, and variability in the fatty acid content of fish consumed within and across populations. In contrast to human epidemiological studies, controlled animal studies, typically conducted in mouse models of human breast cancer,

have demonstrated that a diet supplemented with fish, flaxseed, or canola oil or EPA and/or DHA reduces mammary tumor size, incidence, and multiplicity and decreases the rate of metastasis when compared to a control diet typically containing corn oil, which is high in n-6 PUFA[8-22].

Several independent but interrelated mechanisms are postulated to account for the possible beneficial effects of n-3 in reducing breast cancer risk. Increased intake of n-3 displaces n-6, such as arachidonic acid (AA, 20:4), in cellular membranes. This displacement, in addition to competition for the enzymes cyclooxygenase and lipoxygenase, can shift the production of pro-inflammatory eicosanoids from n-6 to anti-inflammatory eicosanoids from n-3, and this is the primary justification for the use of n-3 in cardiovascular disease prevention[23]. Decreased inflammation may reduce carcinogenesis, cancer progression, and metastases, all processes that are associated with inflammation. Rose et al.[10] measured decreased pro-inflammatory eicosanoids in mice fed either 8% or 4% DHA or EPA versus an equal amount of linoleic acid (LA, 18:2 n-6), and a dietary crossover study showed a significant decrease in plasma F_2-isoprostanes (a marker of AA oxidation) in women consuming 15 g fish oil versus 15 g sunflower oil, providing mostly oleic acid (18:1 n-9), for five weeks[24].

A second potential mechanism for chemoprevention by n-3 is the modulation of signal transduction. Several studies have shown an effect of n-3 supplementation on genes involved in cell cycle progression, apoptosis, lipid metabolism, eicosanoid synthesis, and antioxidant activity[14, 20-22, 25]. It is likely that n-3 affect gene expression through several pathways, but the principle mechanisms appear to be the

alteration of signal transduction by incorporation into cellular membranes and acting as ligands for signal transduction factors, particularly for peroxisome proliferator-activated receptors (PPARs). Displacement of cholesterol and proteins by n-3 in lipid rafts and calveolae within cellular membranes can disrupt receptor-mediated signal transduction, including via the T cell receptor[26]. PUFA and their oxidative metabolites, including 9- and 13-hydroxy-octadecadienoic acid (9- and 13-HODE) have been shown to specifically bind to multiple isoforms of PPARs but especially PPARγ[27, 28]. There is a large body of evidence indicating that PPARγ, which can be over-expressed in tumors including breast cancer, promotes cell differentiation and inhibits inflammation and tumor growth[28-31]. In addition to up-regulation of apoptosis, or programmed cell death, via signal transduction, n-3 consumption may enhance apoptosis from lipid peroxidation. This potential chemopreventive mechanism will be discussed in the following section, Polyunsaturated fatty acids and lipid peroxidation.

Lastly, the role of restricting dietary fat to reduce the risk or severity of breast cancer has been closely examined and must be addressed, although such research does not specifically measure the effects of n-3. In a review commissioned by the World Health Organization and Food and Agriculture Organization, Gerber[32] concluded that there was a probable positive association between total fat intake and risk of breast cancer, likely due to increased estrogen synthesis in the adipose tissue of postmenopausal women. In a large, randomized controlled dietary intervention trial of postmenopausal women diagnosed with early-stage breast cancer entitled the Women's Intervention Nutrition Study (WINS), subjects in the intervention group with a mean daily fat intake

of 33.3 g showed a significant 24% decreased hazard rate for breast cancer relapse compared to subjects in the control group averaging 51.3 g of fat per day after a median follow-up of 60 months[33]. A corollary to WINS was the Women's Healthy Eating Living (WHEL) randomized controlled trial of postmenopausal women diagnosed with operable breast cancer following a low-fat (15-20% of energy), high fiber (30 g), and high fruit and vegetable (eight servings total) diet daily for a mean of 7.3 years[34]. The results of this study showed a modest but non-significant reduction in the hazard rate of breast cancer relapse or overall mortality in the intervention group; the mean energy from fat ranged from 21.2-28.9% during the first 72 months of follow-up in the intervention group. However, the mean energy from fat ranged from 27.8-32.4% in the control group over the same time period, reducing the likelihood of a significant effect of dietary fat given the relatively similar patterns of intake between the two groups. Nevertheless, a decrease in total fat intake may lower the risk of breast cancer by reducing adiposity or through independent mechanisms, suggesting that a low-fat diet (approximately 20% or fewer calories from fat) supplemented with n-3 may be more beneficial than a higher fat diet supplemented with n-3 for breast cancer risk reduction.

Polyunsaturated fatty acids and lipid peroxidation

Chemistry of process and formation of metabolites

Lipid peroxidation refers to the free radical-initiated autocatalytic degradation of unsaturated fatty acids in the presence of molecular oxygen[35]. The rate of lipid peroxidation increases exponentially with an increasing number of double-bonds, and

highly unsaturated fatty acids such as DHA and EPA are exceedingly susceptible to lipid peroxidation[36, 37]. Following the removal of a hydrogen from a methylene group (—CH_2—) between the double bonds of the fatty acid by a free radical, such as the hydroxyl radical (·OH) (Figure 2), the resulting lipid radical undergoes conformational changes and reacts with molecular oxygen to yield a peroxyl radical (LOO·) followed by a lipid hydroperoxide (LOOH) (Figure 3 and Figure 4)[38]. This unstable lipid peroxyl radical is converted to a lipid alkoxy radical coupled to the production of an additional hydroxyl radical (Figure 5), which readily degrades via β-scission into several reactive metabolites (Figure 6)[35].

Several reactive electrophilic compounds are formed following the β-scission of lipid peroxyl radicals, including malondialdehyde (MDA), 4-hydroxyalkenals, 2-alkenals, and 2,4-alkadienals[35, 36]. Of these metabolites, the 4-hydroxyalkenals, 4-hydroxy-2-*trans*-nonenal (HNE) and—to a lesser extent—4-hydroxy-2-*trans*-hexenal (HHE), in addition to MDA, are the most extensively studied (Figure 7)[35, 38-40]. It has been shown that HNE is produced from the lipid peroxidation of both n-6 and n-3, whereas HHE is generated almost exclusively from n-3[36, 41, 42].

Because they contain multiple double bonds, EPA and DHA are considered highly susceptible to oxidative stress. Indeed, Guillen et al.[43] measured a number of lipid peroxidation products in the head space of cod liver oil, including ketones, monounsaturated aldehydes, diunsaturated aldehydes, and oxygenated aldehydes. A lipidomic analysis of DHA methyl ester by Kawai et al.[44] established that

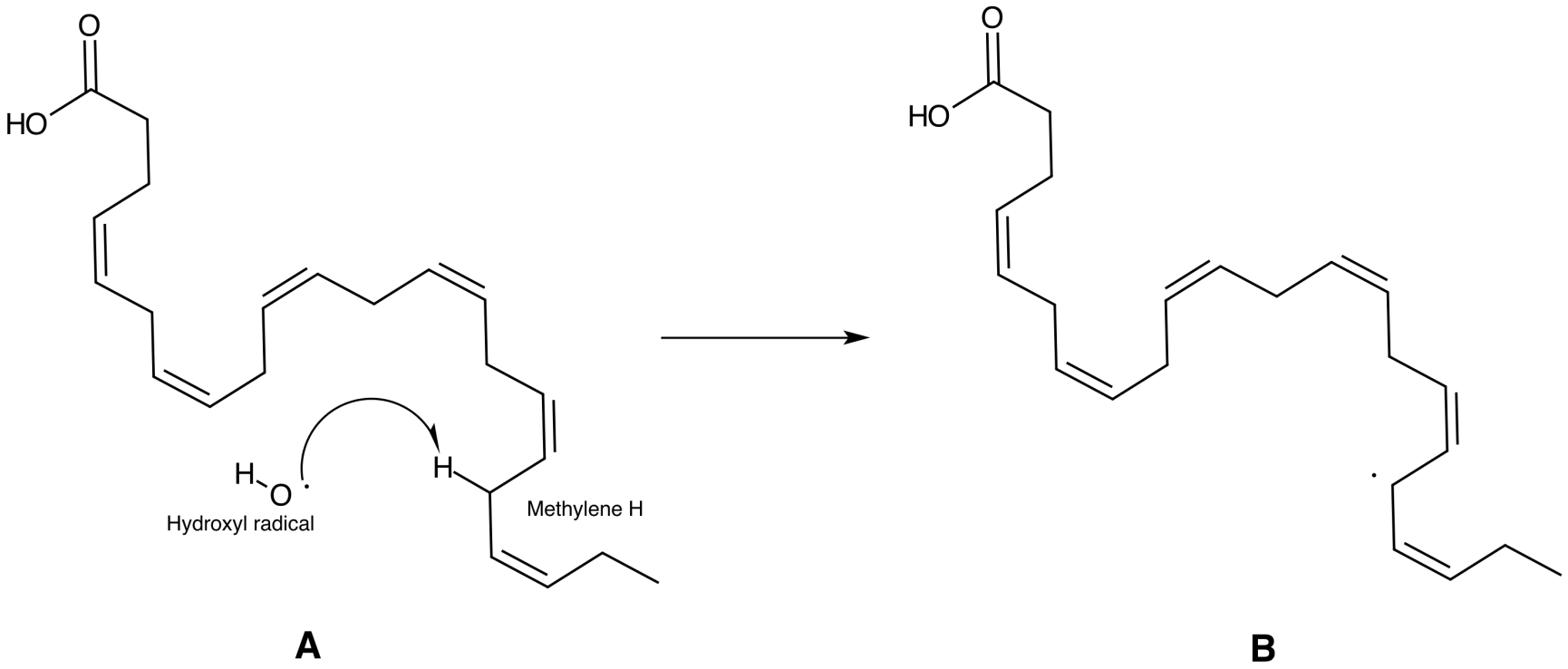


Figure 2. Initiation of lipid peroxidation by free radical attack on methylene hydrogen of the n-3 docosahexaenoic acid (DHA, 22:6, A) and conversion to DHA radical (B) (adapted from [3]).

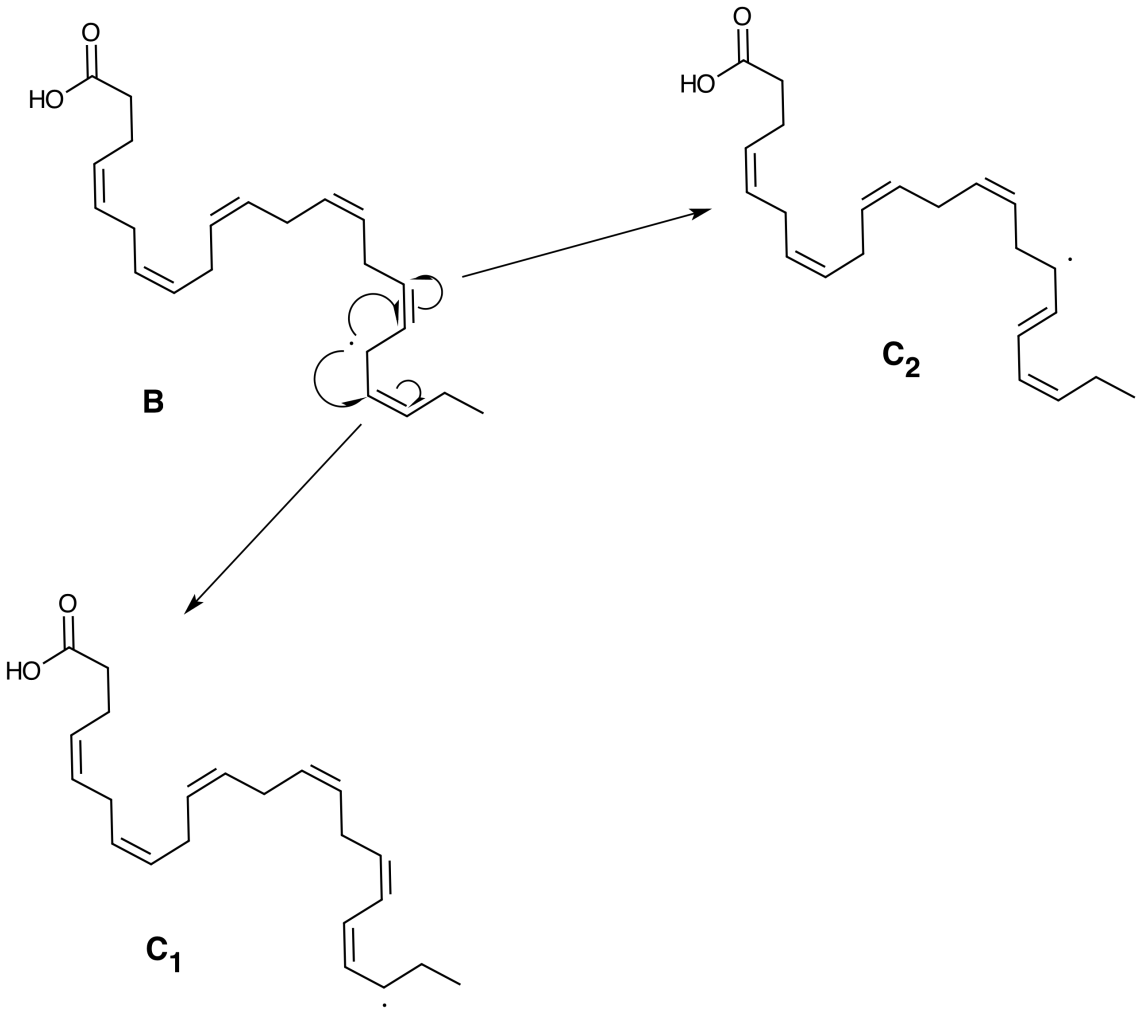


Figure 3. Formation of conformational structures (C_1 and C_2) from DHA radical (B) (adapted from [3]).

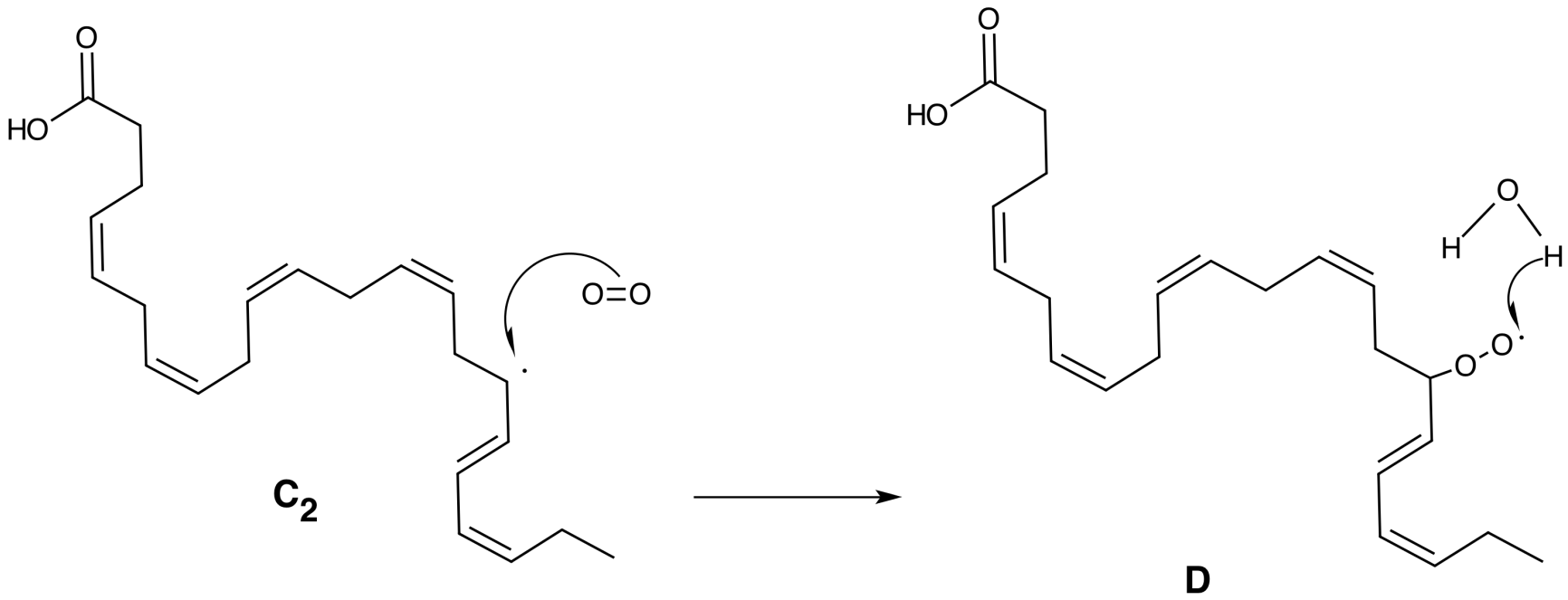


Figure 4. Reaction of DHA radical (C_2) with molecular oxygen to form DHA lipid peroxyl radical (D) (adapted from [3]).

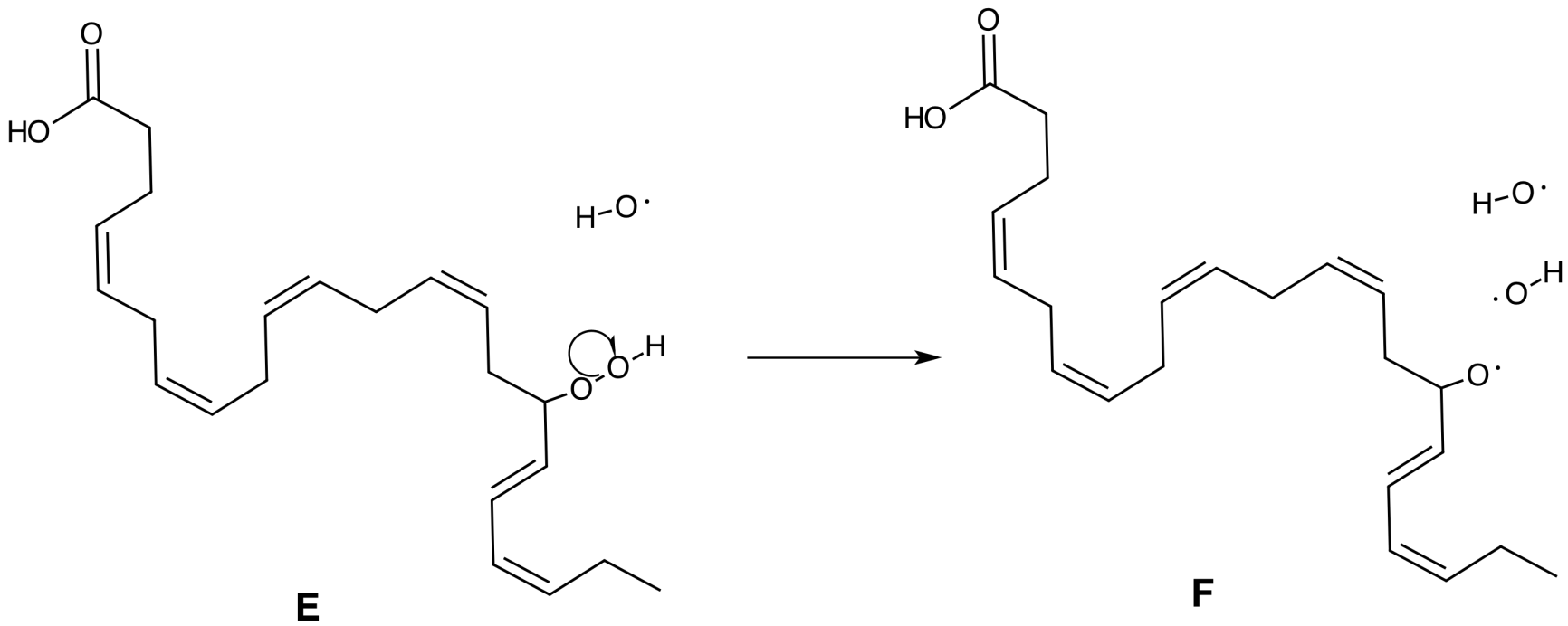


Figure 5. Degradation of DHA lipid peroxide (E) into DHA alkoxy radical (F) coupled to production of additional hydroxyl radicals (adapted from [3]).

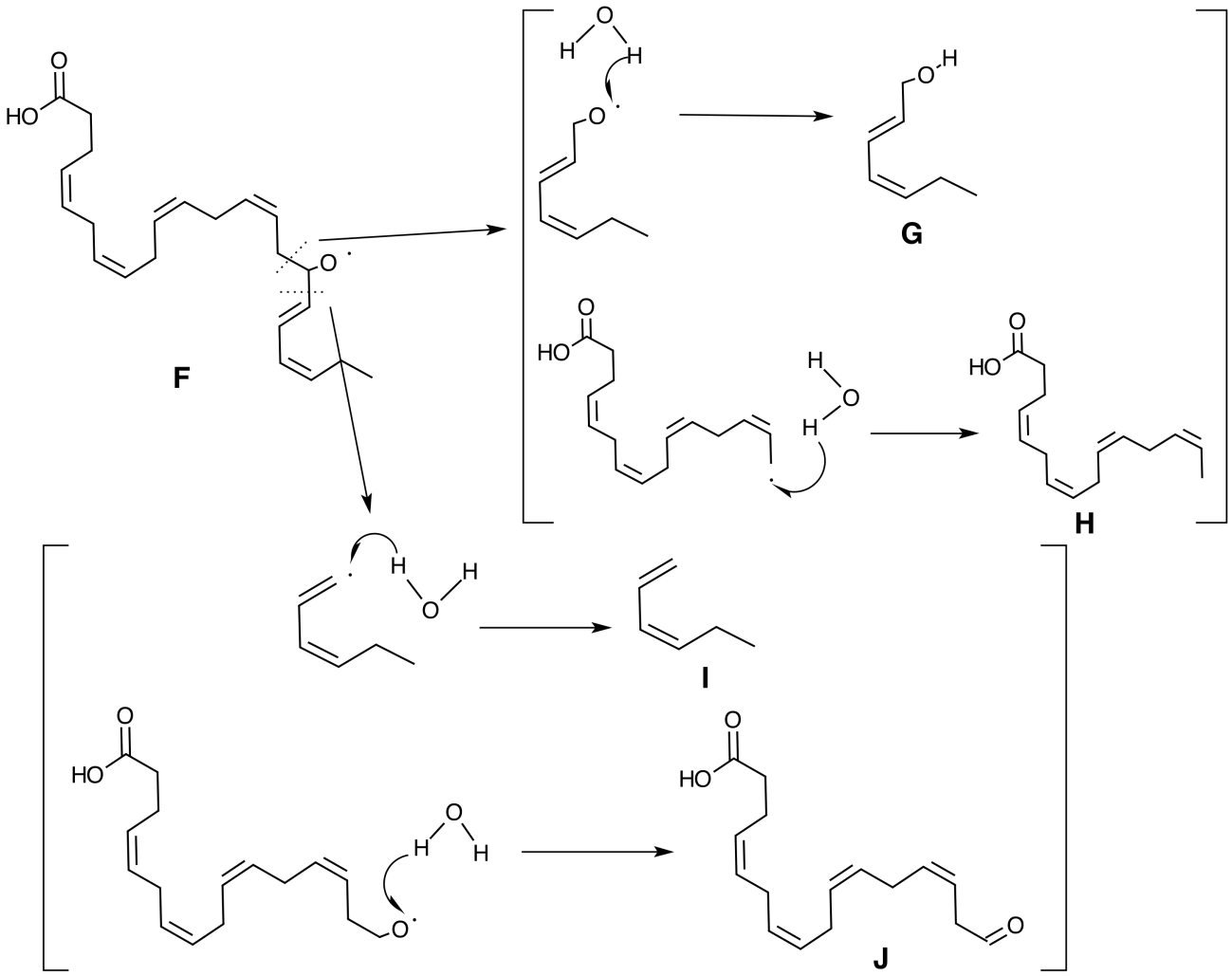
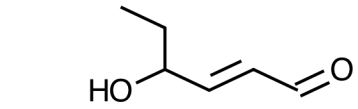
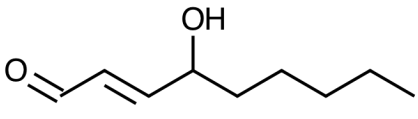


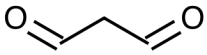
Figure 6. Degradation of DHA alkoxy radical (F) via β-scission into aldehyde (G), acid (H), hydrocarbon (I), and/or oxoacid (J) (adapted from [3]).



4-Hydroxy-2-*trans*-hexenal (HHE)



4-Hydroxy-2-*trans*-nonenal (HNE)



Malondialdehyde (MDA)

Figure 7. Common reactive metabolites formed from lipid peroxidation of polyunsaturated fatty acids (PUFA).

4-oxo-butanoate, 2-pentenal, 2-octenal, 2,4-heptadienal, 8-oxo-4,6-octadienoate, 2-hydroxybutanal, 5-oxo-4-hydroxypentanoate, HHE, 4-hydroxy-2,6-nonadienal, 7-oxo-4-hydroxy-5-heptenoate, 4-hydroxy-2,6,9-dodecatrienal, 10-oxo-7-hydroxy-4,8-decadienoate, 13-oxo-10-hydroxy-4,7,11-tridecatrienoate, glyoxal, and MDA were detected with gas chromatography-mass spectrometry following the induction of oxidation.

Research examining the production of specific lipid peroxidation metabolites following fish or long-chain PUFA consumption by humans is presently lacking. However, the measurement of thiobarbituric acid reactive substances (TBARS) in plasma or urine is commonly employed as a non-specific measurement of lipid peroxidation[45]. Table 1 summarizes relevant studies examining the response of *in vivo* lipid peroxidation to dietary supplementation with fish oil and/or n-3. In general, fish oil or n-3

Table 1. Summary of human intervention studies of fish oil and/or n-3 PUFA and *in vivo* lipid peroxidation.

Reference	Subjects	Intervention	Peroxidation Outcome Measurement(s)	Results	Notes
Meydani et al.[46]	Human female, 22-35 or 55-71 y n=25	Daily supplement for 3 months: • 1680 mg EPA • 720 mg DHA • 600 mg other fatty acids 6 IU vitamin E	Plasma fatty acids Plasma vitamin E Plasma TBARS	Older women had sig. higher increase in plasma EPA and DHA than younger women. Sig. increase in ratio of PUFA:saturated fatty acids in both age groups with supplementation. Sig. decrease in plasma triglycerides in both age groups with supplementation. Sig. decrease in plasma vitamin E in young women at 3 month compared to 1 month of supplementation. Sig. decrease of vitamin E relative to EPA and DHA in both age groups with supplementation. Plasma MDA equivalents sig. higher at month 2 compared to baseline in both age groups, but no significant difference relative to baseline at 3 months.	Decreased MDA at 3 months possibly due to increased activity of GSTs. Female hormones reported to have antioxidant activity.
Nair et al.[47]	Human men, 24-57 y n=40	Controlled diet with: • 15 g/d of placebo oil for 10 weeks • 15 g/d fish oil concentrate with 15 mg vitamin E for 10 weeks • 15 g/d fish oil concentrate with 200 mg vitamin E for 8 weeks	Plasma TBARS Plasma α-tocopherol, retinol, and β-carotene	Plasma TBARS sig. increased with fish oil diet but sig. decreased with fish oil + vitamin E compared to the placebo diet. Sig. decrease of plasma α-tocopherol with fish oil diet, restored to placebo level with 200 mg vitamin E.	

Reference	Subjects	Intervention	Peroxidation Outcome Measurement(s)	Results	Notes
Suzukawa et al.[48]	Human, hypertensive men (n=6) and women (n=14) (mean age 60 y)	6 wk. crossover: • 3.4 g/d n-3 fatty acids + 2 mg/mL vitamin E • 3.4 g/d corn oil + 2.2 mg/mL vitamin E	Plasma TBARS Plasma lipid peroxides LDL TBARS Copper-induced LDL oxidation	No sig. difference in plasma lipid peroxides between 2 supplement periods or baseline. Sig. reduction in lag time with fish oil supplementation for copper-induced LDL oxidation, particularly among subjects taking both a diuretic and beta-blocker. Fish oil treatment sig. increased LDL TBARS compared to baseline or corn oil treatment with 90 or 180 minutes oxidation. TBARS also increased with fish oil treatment during macrophage culture experiment.	Authors conclude there is a need for increased α-tocopherol intake with large doses of fish oil.
Palozza et al.[49]	Human male (n=22) and female (n=18) (25-46 y)	Double-blind randomized controlled dietary trial for 6 mos. of: • 2.5 g/d PUFA + 2.7 mg vitamin E • 5.1 g/d PUFA + 2.7 mg vitamin E • 7.7 g/d PUFA + 2.7 mg vitamin E Placebo with mixed fatty acids and no added vitamin E	2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH)-induced lipid peroxidation of plasma membranes followed by TBARS	Dose-dependent increase in TBARS, sig. in highest dose of PUFA for 6 months.	
Wander et al.[45]	Human female, postmenopausal n=48	Double-blind crossover trial for 5 wks: • 15 g/d fish oil + 0 mg vitamin E	Urinary TBARS Urinary and plasma MDA by HPLC	Sig. increase in urinary TBARS with fish oil vs. baseline, which decreased with increasing amounts of vitamin E. Sig. increase of urinary MDA with fish oil	

Reference	Subjects	Intervention	Peroxidation Outcome Measurement(s)	Results	Notes
		• 15 g/d fish oil + 100 mg vitamin E • 15 g/d fish oil + 200 mg vitamin E • 15 g/d fish oil + 400 mg vitamin E		not ameliorated by vitamin E. Sig. increase of plasma MDA with fish oil supplement, not sig. decreased with vitamin E.	
Turley et al.[50]	Human female (18-28 y) n=30	Double-blinded, parallel-arm, 28 d supplement with: • 2.4 g fish oil 2.4 g fish oil + 3 mg/g vitamin E	Plasma ascorbate Plasma MDA and HNE	Borderline sig. decrease in plasma ascorbate in fish oil group, and borderline sig. increase in fish oil + vitamin E group. No sig. differences in plasma MDA and HNE from baseline or between groups.	
Saito et al.[51]	Hyperlipidemic female (48-58 y) n=5	2-wk. controlled diet with: • 2.43 g/d EPA	Plasma TBARS Water-soluble fluorescent substances	No sig. changes in plasma TBARS and sig. decrease in water-soluble fluorescent substances following EPA supplementation.	Authors recommend double the equation 0.3 x double-bond index x oil supplement intake (g) to ensure adequate vitamin E.
Higdon et al.[24]	Human female, postmenopausal (50-75 y) n=15	3-treatment crossover trial (5 wks. each): • 15 g sunflower oil (12.3 g oleate) • 15 g safflower oil (10.5 g linoleate) • 15 g fish oil (2.0 g EPA and	Plasma F_2-isoprostanes Plasma MDA Plasma TBARS	After fish oil treatment, plasma MDA 17% lower (sig.) than sunflower oil and 13% lower (NS) than safflower oil. After fish oil treatment, plasma TBARS were more than 21% higher than sunflower oil and 23% higher than safflower oil treatment (both sig.). No difference in MDA or TBARS between sunflower and safflower supplementation periods.	

Reference	Subjects	Intervention	Peroxidation Outcome Measurement(s)	Results	Notes
		1.4 g DHA)		Normalization of MDA to plasma PUFA eliminated sig. differences between diets, but normalization of TBARS to PUFA maintained sig. higher concentration with fish oil supplementation. F2-isoprostanes sig. lower following fish oil treatment than sunflower oil.	
Grundt et al.[52]	Human male (n=238) and female (n=62) with recent myocardial infarction 28-87 y	Double-blinded, placebo-controlled, randomized trial for 1 y: • 3.464 g/d n-3 • 4 g/d corn oil	Serum MDA via HPLC	Sig. increase in TBARS in fish oil group vs. baseline or corn oil group.	
Wu et al.[53]	Human female vegetarian, postmenopausal n=27	Single-blind, randomized, placebo-controlled trial for 6 wks. of: • 6 g/d DHA-rich algae oil (2.14 g/d DHA, 1 IU vit. E/g) • 6 g/d corn oil	LDL TBARS following copper-induced oxidation Urinary 8-iso-prostaglandin F_2	Sig. increase in LDL TBARS with DHA supplementation. No difference in urinary F_2 isoprostane between groups.	
Calzada et al.[54]	Human male, 53-65 y n=12	Increasing doses of DHA for 2 weeks each: • 200 mg + 0.125 mg vitamin E • 400 mg + 0.25 mg vitamin E • 800 mg + 0.375 mg vitamin E • 1600 mg + 0.5 mg vitamin E	LDL vitamin E LDL MDA via HPLC LDL and cholesteryl ester fatty acids Copper-induced LDL oxidation Plasma hydroxy-alkenals	Sig. increase in LDL vitamin E with all supplementation except 1600 mg DHA, highest increase with 200 mg. Sig. decrease in LDL MDA with all supplementation except 1600 mg. Sig. increase in lag time for copper-induced LDL oxidation in 200-800 mg DHA, highest time with 400 mg. Sig. increase in plasma HHE with 800 and 1600 mg DHA.	HHE represented 0.01% of plasma n-3 PUFA.

Reference	Subjects	Intervention	Peroxidation Outcome Measurement(s)	Results	Notes
Kimura et al.[55]	Human male (n=290) and female (n=205)	Cross-sectional	Serum fatty acid composition Urinary 8-oxoGua	Sig. positive correlation for serum n-3 PUFA, EPA, and DHA with urinary 8-oxoGua. Sig. positive correlation between double bond index and urinary 8-oxoGua.	8-oxoGua is a marker of oxidative DNA damage.

Abbreviation:
8-oxoGua = 8-oxo-7,8-dihydroguanine

supplementation increases plasma, low-density lipoprotein (LDL), or urinary TBARS relative to baseline or control groups[24, 45-49, 52, 53]. It is notable that increased vitamin E supplementation was shown to ameliorate the increase in TBARS from high n-3 intake in two of the studies[45, 47]. In contrast to the TBARS results, a more specific measurement of plasma or LDL cholesterol MDA via high-performance liquid chromatography (HPLC) indicated no differences or decreased concentrations following fish oil supplementation[24, 50, 54] with the exception of Wander et al.[45]. Additionally, in a study of men only, Calzada et al.[54] showed a significant increase in plasma HHE following DHA supplementation at 800 or 1600 mg per day, which comprised 0.01% of total plasma n-3 PUFA. However, Turley et al.[50] did not detect a significant increase of plasma HNE with daily fish oil supplementation in young women compared to baseline or women consuming vitamin E in addition to fish oil.

Biological effects of metabolites

In contrast to free radicals, aldehydic degradation products of lipid peroxidation are relatively stable and believed to diffuse from the site of production to distal tissues within the body[39]. 4-Hydroxyalkenals possess an α,β-unsaturated structure that renders the compounds electrophiles and are readily bound via Michael addition by nucleophiles such as DNA, proteins, and phospholipids[56]. Such binding can cause a number of deleterious effects including mutagenesis, enzyme inhibition, and cell death.

In 1984, Chung et al.[57] demonstrated that acrolein, an α,β-unsaturated aldehyde found in both foods and cigarette smoke, can form adducts with deoxyguanosine *in vitro*.

These researchers further detected DNA adducts from additional lipid peroxidation metabolites, such as HNE, in the liver and colon tissues of rats and humans[58, 59]. However, in a mouse model of tumor induction, HNE or its epoxide (at 50, 100, or 200 nmol injected intraperitoneally) failed to induce lung or liver tumors at statistically higher rates than vehicle controls; the epoxide—but not HNE—did induce skin papilloma development at significantly higher incidences than acetone control when applied to the skin of mice at 128 μmol[60]. The inability of HNE to promote tumorigenesis was attributed to the well-documented repair of DNA adducts by DNA glycosylase[58, 61]. Nevertheless, DNA adducts with the oxidized product of HHE, 4-oxo-2-*trans*-hexenal (4-OHE), have been detected recently in noncancerous lung tissues from lung cancer patients, independent of age and smoking status[62]. More recently, serum n-3 PUFA, EPA, and DHA were significantly positively correlated to urinary 8-oxo-7,8-dihydroguanine (8-oxoGua) in a Japanese population, suggesting an association between dietary n-3 intake and oxidative DNA damage[55].

Similar to their reaction with DNA, 4-hydroxyalkenals can modify proteins through Michael addition by binding to the sulfhydryl group of cysteine[35, 63] and imidazole nitrogens of histidine and lysine[40, 64, 65]. It has also been established that HNE can bind to a terminal lysine via Schiff base formation[40, 65, 66]. Protein binding by high concentrations (25 μM to 1 mM) of HNE has been shown to substantially reduce the enzymatic activity of glucose-6-phosphate dehydrogenase[67], cytochrome P450 (CYP) 2E1, 3A6, and 2B4[68, 69], and glyceraldehyde-3-phosphate dehydrogenase, potentially impairing the biological pathways of glycolysis and detoxification[70, 71]. Additionally,

HNE and other aldehydes produced during lipid peroxidation were shown to bind via Michael addition to bovine serum albumin, and HNE bound carnosine, a peptide found in cardiac and skeletal muscle tissue[72, 73]. Binding of the latter peptide is considered a detoxification mechanism, but binding to serum albumin may explain how HNE and other lipid peroxidation metabolites are able to travel through the bloodstream.

Finally, HNE has been shown to result in the death of cells via necrosis or apoptosis[39]. Conditions of oxidative stress, as would lead to lipid peroxidation, are known to activate the tumor suppressor protein p53, resulting in the mitochondrial process of apoptosis[74]. Pillon et al.[75] showed that HHE, HNE, and other lipid peroxidation metabolites at high concentrations (100 μM) result in more necrosis than apoptosis of muscle cells, which the authors attributed to lethal binding to proteins within plasma membranes or organelles; however, apoptosis was also detected in the treated cells from a significant increase in caspase-3 activity. The cytotoxic activity of HNE has also been attributed to its alteration of cell signaling pathways[76, 77].

Role of antioxidants

Oxidative stress and lipid peroxidation can be quelled by exogenous or endogenous antioxidants[78]. Of the exogenous dietary antioxidants, the lipid-soluble vitamin E is the most important for suppressing lipid peroxidation[1]. Several studies have shown that an increase in dietary PUFA must be accompanied by an increase in dietary vitamin E to decrease lipid peroxidation[45, 79, 80]. It has been established that vitamin C can quench free radicals, and there is some evidence that it may inhibit lipid peroxidation,

either directly or via regeneration of vitamin E from the vitamin E radical[1, 81, 82]. Non-essential dietary antioxidants, such as soy isoflavones, may also reduce lipid peroxidation[83].

Endogenous antioxidant systems are directly involved in the quenching of free radicals as well as lipid hydroperoxides. Although there are no inherent antioxidant enzymes that consume the hydroxyl radical, superoxide dismutase (SOD) converts two superoxide molecules (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (Figure 8), which prevents the formation of the hydroxyl radical via the Haber-Weiss reaction (Figure 9)[84, 85]. Hydrogen peroxide can be converted into water by catalase, peroxiredoxins, or glutathione peroxidase (GPx)[84]. GPx, which contains the essential nutrient selenium, can also detoxify lipid hydroperoxides into hydroxy fatty acids[86].

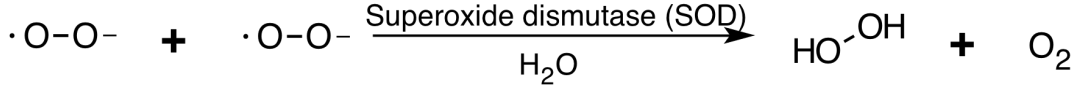


Figure 8. Conversion of two superoxide molecules (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) via the enzyme superoxide dismutase (SOD).

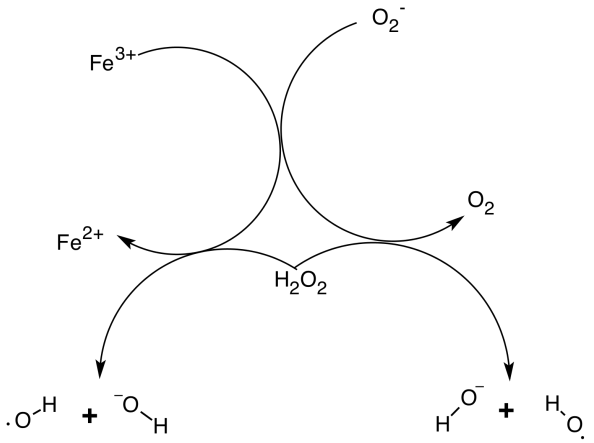


Figure 9. Haber-Weiss reaction cycle.

Detoxification and excretion of metabolites

In addition to the aforementioned endogenous antioxidant enzymes, several metabolic pathways serve to detoxify and excrete lipid peroxidation metabolites from the body. Aldehyde dehydrogenases (ALDH) are ubiquitously expressed in human tissues and function to oxidize aldehydes, such as those produced during lipid peroxidation, into carboxylic acids[87]. It has been proven in humans and rats that HNE is readily oxidized to 4-hydroxy-2-*trans*-nonenoic acid (HNA) by ALDHs[69]. However, the primary detoxification mechanism in mammals appears to be reduction of HNE by alcohol dehydrogenases (ADHs) or aldo-keto reductases (AKRs) to 1,4-dihydroxy-2-*trans*-nonene (DHN) (Figure 10)[88-90].

Reactive electrophiles, such as HNE and HNA, can be bound to the tripeptide glutathione (GSH), with or without catalysis by glutathione-*S*-transferases (GSTs)[89]. DHN, which no longer possesses an α,β-unsaturated structure, cannot form adducts with GSH; however, HNE adducted to GSH can be reduced to form DHN-GSH. Binding with GSH can occur in all cell types, but the liver is the site of the greatest frequency[91]. Upon formation, GSH adducts are readily transported to the kidneys, where they are catabolized to cysteine S-conjugates and N-acetylated to form mercapturic acids excreted in the urine. Mercapturic acids may also be formed in the liver and excreted into the bile or circulation, but urinary mercapturic acids offer an opportunity for non-invasive measurement of the excretion of electrophilic compounds formed endogenously, present in the diet, or from exposure to environmental toxins. Mercapturic acid conjugates of DHN and HNA have been detected in the urine of rats and humans, and this pathway

represents the accepted mechanism for 4-hydroxyalkenal detoxification and excretion in mammals[82, 88-90].

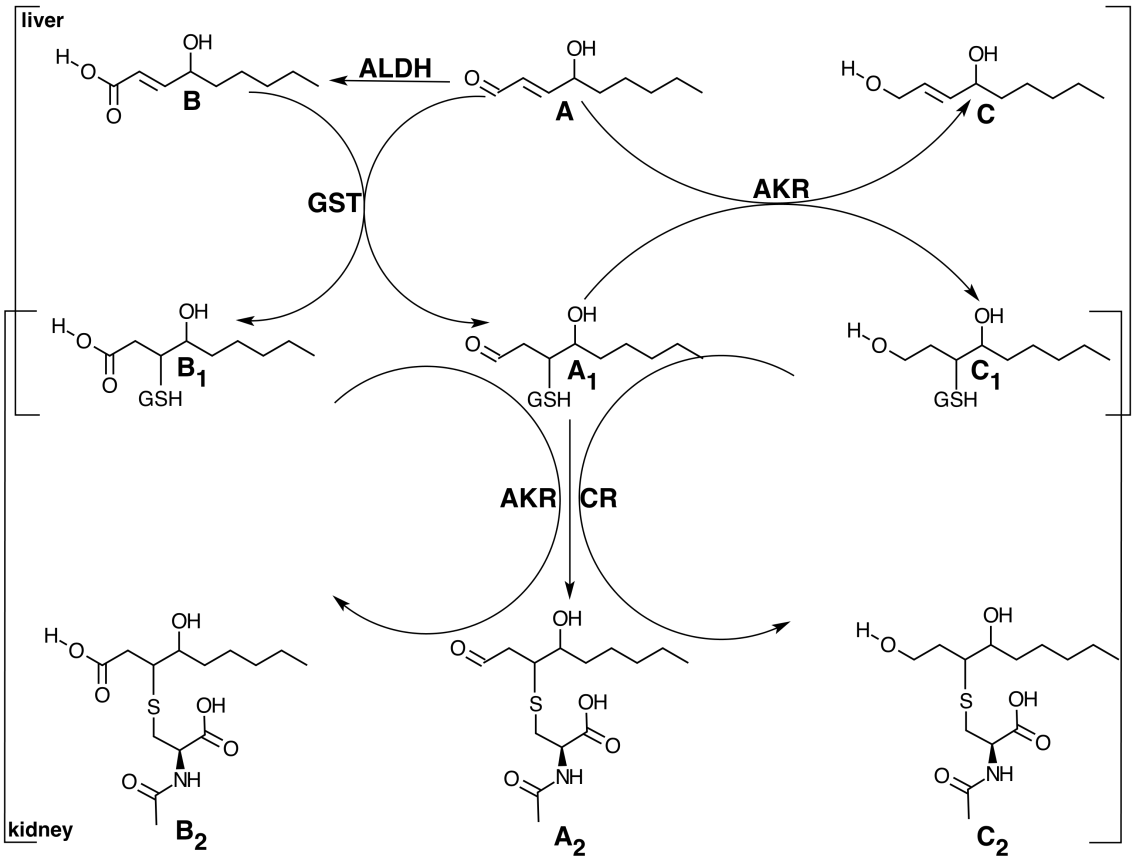


Figure 10. Metabolic conversion of the α,β-unsaturated hydroxyaldehyde, 4-hydroxy-2-*trans*-nonenal (HNE, A), via aldehyde dehydrogenase (ALDH) into 4-hydroxy-2-*trans*-nonenoic acid (HNE, B) or via aldo-keto reductase (AKR) into 1,4-dihydroxy-2-*trans*-nonene (DHN, C) in the liver. A and B can form glutathione (GSH)-conjugates (A_1 and B_1), typically catalyzed by GSH–*S*-transferase (GST); alternatively A may bind to GSH followed by reduction via AKR to form the GSH-conjugate of DHN (C_1). The GSH-conjugates are catabolized to mercapturic acids (A_2, B_2, and C_2) via AKR or carbonyl reductase (CR) in the kidney for urinary excretion (adapted from [89]).

Free radicals, lipid peroxidation, and cancer

As mentioned previously, free radicals and electrophilic lipid peroxidation metabolites may bind to DNA, leading to genetic instability or mutations. Such modifications are implicated in cancers, which are characterized by DNA mutations resulting in cellular immortality, sustained proliferation, evasion of apoptosis and immune detection, the promotion of new blood vessel formation, and the ability to spread to and invade distal sites with the organism[61, 92]. Conditions of elevated oxidative stress have been detected in case-control studies of cancer patients, but it has been difficult to ascertain the role of oxidative stress in carcinogenesis[61]. The subsequent section includes an extensive review of primary literature regarding lipid peroxidation and breast cancer.

It has been estimated that oxidative lesions to DNA are present under steady-state conditions at 0.4-0.8 clusters per million base pairs, and HNE is present within cells under steady-state conditions at 5-10 μM[61, 73]. Consequently, endogenous DNA repair activity and antioxidants have important functions to maintain homeostasis and prevent the formation of tumors. In addition to the aforementioned antioxidant and detoxification pathways to eliminate free radicals and reactive electrophiles, base excision repair and nucleotide excision repair serve to repair DNA damage resulting from oxidative modifications and prevent genetic mutations that may lead to cancer[73]. Nevertheless, the disruption of DNA repair or antioxidant activity with or without an increase in cellular exposure to free radicals would promote mutagenesis in a typical cell.

Cellular oxidative stress also plays an important role in the function of a key tumor

suppressor protein, p53. The gene *p53*, also called *TP53*, is frequently mutated in human cancers[93]. When intact, p53 can induce the mitochondrial pathway of apoptosis in response to oxidative stress[74]. At low levels of oxidative stress, p53 serves as a transcription factor to increase the expression of the antioxidant enzymes SOD and GPx. The protein itself senses the redox status of cells via ten cysteine residues located in its DNA-binding domain; oxidation of these residues leads to the formation of disulfide bonds within the protein, resulting in a conformational change that may allow p53 to bind to its response elements on DNA. When functional, p53 acts to protect the cell against oxidative stress by up-regulation of antioxidant enzymes or the stimulation of apoptosis. However, if p53 is mutated in cancer, it no longer protects the cell and cannot stave off the increased oxidative stress and replicative potential present in cancer.

In the event of an oncogenic transformation of a cell that is not repaired or eliminated via apoptosis, there is evidence that oxidative stress enhances the promotion and progression of cancer. If they do not trigger apoptosis or necrosis, free radicals can serve to promote the development of cancers by acting as second messengers[94]. Cell culture work has demonstrated that reactive oxygen species (ROS) are involved in cell cycle progression through the Ras/ERK/MAPK pathway, which can be mutated in human cancers[94, 95]. Oxidative stress is also known to activate NF-κB, a transcription factor that promotes cell proliferation[94, 96]. In the later stages of cancer, ROS participate directly in angiogenesis, invasion, and metastasis by disrupting structural proteins or by increasing the expression of and activating matrix metalloproteases (MMPs), enzymes that remodel extracellular matrices allowing for the formation of new blood vessels and

distal transport of tumor cells[94].

Recently, Cortes et al.[97] used RNA microarray to assess changes in breast cancer cells and normal breast cells following the induction of oxidative stress; the transformed cells were relatively unaffected compared to the normal cells. Notably, genes involved in the metabolism of antioxidants were up-regulated in the normal cells but not the cancer cells. Additionally, genes regulating DNA repair were up-regulated by oxidative stress. The authors were surprised that oxidative stress tended to down-regulate the expression of cancer progression genes in all three cell lines tested but were able to differentiate and correlate those genes responding to the oxidative stress versus those responding to cancer progression. *SOD* and *GPx* were up-regulated by both oxidative stress and cancer progression, while *GSTs* and genes encoding for peroxiredoxins were up-regulated by oxidative stress but down-regulated by cancer progression. Therefore, it is evident that an important part of cancer progression is the change in antioxidant enzyme expression patterns, allowing for either the promotion or suppression of malignancies in response to pro-oxidative conditions.

Lipid peroxidation and breast cancer

Cell culture and in vitro *studies*

As summarized in Table 2, several primary research articles have examined n-3 and lipid peroxidation in human breast cancer cell lines. There is general agreement that treating such cells with PUFA including n-3 increases lipid peroxidation measured via TBARS and decreases cell viability. Begin et al.[98] tested DHA and EPA in addition to

Table 2. Summary of cell culture studies examining lipid peroxidation and breast cancer.

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
Begin et al.[98]	Human breast cancer cells ZR-75-1 Untreated cancer cells as controls	20 μg/mL gamma linolenic acid (GLA), arachidonic acid, DHA, EPA	Cell viability TBARS Phospholipid composition	GLA-induced cytotoxicity suppressed by SOD. DHA was least effective and EPA intermediate in increasing TBARS. GLA and AA generated highest levels of TBARS. The addition of iron or copper increased GLA cytotoxicity, whereas vitamin E, BHT, and BHA suppressed cell death. Increase in TBARS correlated with increase in cell death.	
Menendez et al.[99]	Human breast cancer cells SK-Br3 and MDA-MB-231 (negative control because constitutive expression of fatty acid synthase) Untreated cancer cells as controls	0, 6.25, 12,5, 25, or 50 μg/mL LA, GLA, AA, ALA, EPA, and DHA	Cell viability *In situ* immunofluorescent staining of fatty acid synthase (FAS)	ALA sig. decreased cell viability. FAS over-expression and activity in breast cancer cells sig. suppressed by ALA and GLA (strongest effect), and FAS activity sig. reduced by LA, DHA, and EPA. FAS over-expression suppression by ALA and GLA inhibited by vitamin E. FAS expression reduced with MAPK and PI-3'K inhibitors in SK-Br3 cells.	GLA is n-6. Authors conclude effect of PUFA on cell growth due to functions in addition to lipid peroxidation (gene expression).
Maheo et al.[100]	Human breast cancer cells MDA-MB-231, MCF-7, and MCF-7dox (doxorubicin-resistant) Untreated cancer cells as controls	30 μM DHA	Cellular phospholipid composition Cell viability MDA, GSH, GSSG, and vitamin E concentrations via HPLC-UV	DHA incorporated sig. more in MDA-MB-231 than MCF-7 membranes. DHA sig. increased cytotoxicity of doxorubicin in MDA-MB-231 and MCF-7dox cells. DHA increased MDA concentration by approx. 4-fold in each cell line. GSH and GSSG sig. increased by DHA in MDA-MB-231 cells. Vitamin E levels were undetectable.	
Menendez et al.[101]	Human breast cancer cells BT-474 and SKBr-3	0, 2.5, 5, 10, 20, or 40 μM ALA	HER2 quantification via ELISA, immunoblotting, and *in situ* immunofluorescent	ALA treatment sig. reduced HER2 protein levels and *HER2* gene expression in both cell lines.	HER2 overexpression associated with

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
	Untreated cancer cells as controls		staining Cell cycle distribution via flow cytometry *HER2* gene concentration via RT-PCR HER2 promoter activity Cell viability	ALA treatment sig. enhanced reduction in cell viability with trastuzumab treatment.	aggressive breast cancers, decreased survival times, and increased relapse rates. LA shown to increase HER2 expression.
Grossman et al.[102]	Human breast cancer cells MDA-MB-231 (ER-negative) and MDA-ERα7 (ER-positive) Untreated cancer cells as controls	0, 2.5, 5, 10, 20, 40, or 80 μM eleostearic acid (ESA) (conjugated LA), CLA, or ALA	Cell proliferation Immunoblotting and Western blots of poly ADP ribose polymerase (PARP) Cell cycle distribution via flow cytometry Apoptosis via flow cytometry kit Mitochondrial membrane potential via kit	20-80 μM ESA sig. decreased proliferation of both cell lines and sig. increased apoptosis. These effects were eliminated by the addition of vitamin E. Similar results seen for mitochondrial membrane potential, indicating ESA enhanced intrinsic apoptotic pathway. No effect of ESA on cleaved PARP concentrations. ESA treatment sig. increased proportion of cells in G_2-M phase. Inhibition of AMPK blocked the effect of ESA on cell proliferation.	ESA found in bitter melon seed.
Cipak et al.[103]	Human breast cancer cells SUM159 Untreated cancer cells as controls	Treatment with hydroxyl radical Treatment with 10, 35, or 50 μM HNE	Differential scanning calorimetry Cellular dichroism Immunoblotting for HNE-collagen and protein adducts Cell viability	Treatment of collagen with hydroxyl radical produced two denaturation transitions, causing significant structural changes. Treatment of cells with native collagen decreased cytotoxicity of HNE, whereas oxidized collagen + HNE sig. decreased viability. Cells with oxidized-collagen had greater amounts of HNE-protein adducts than those with native collagen.	HNE above 10 μM supraphysiological. Oxidative treatment decreased amino N content due to modification of lysines. HNE has high affinity for histidine, lysine, cysteine, and arginine. HNE binds in particular to membrane-

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
					associated proteins.
Rysman et al.[104]	Human cancer cells, including breast cancer cells BT474 Untreated cancer cells as controls Human prostate cancer tissues with matching normal tissues	siRNA targeting FAS and acetyl-CoA carboxylase (ACC)	Cellular phospholipid composition Lipid peroxidation product kit (MDA and/or HNE) Immunoblotting for FAS and ACC concentrations Lateral membrane dynamics via fluorescence recovery and diffusion Membrane flip-flop via fluorimeter Cell death	Inhibition of ACC sig. increased membrane PUFA and decreased saturated fatty acids and MUFA, particularly in phosphatidylcholine. Prostate cancer tissues with increased FAS expression also had increased saturated and MUFA and decreased PUFA compared to control tissues. Inhibition of ACC sig. increased lipid peroxidation products, which was reversed with supplemental palmitic acid. Inhibition of ACC also enhanced cell death due to hydrogen peroxide, which was also suppressed by palmitic acid. Addition of PUFA increased lipid peroxidation-induced cell death with ACC inhibition. FAS inhibition shown to increase lateral and flip-flop membrane mobility. ACC inhibition enhanced cell death from doxorubicin.	Increased membrane saturated fatty acids decreased lipid peroxidation-induced cell death and entry of chemotherapeutics into cancer cells.
Slade et al.[105]	Human breast cancer cells MCF-7 Untreated cancer cells as controls *In vitro* cytochrome c binding	64 μM 13-HPODE (LA hydroperoxide) and ascorbic acid or 13-HPODE-biotin and ascorbic acid	Immunostaining Neutravidin affinity chromatography 2D-SDS-PAGE and Western blot to identify modified proteins Mass spectrophotometry to identify modified proteins	Treatment with 13-HPODE results in DODE binding to cytochrome c. 98 proteins identified as modified by DODE; 31 identified by all 3 techniques. Most proteins were cytoplasmic, but 17% were nuclear. Main protein groups were molecular chaperones, glycolysis, regulatory, nucleotide binding, and structural.	Biotin modification allows for better uptake by cells. Extraction used not optimal for preserving membrane proteins.
Cortes et al.[97]	Human breast cancer cells HMLER-1 and	0, 0.2 units of glucose oxidase	GSH and GSSG concentrations via kit RNA microarray	11,895 genes differentially expressed in response to oxidative stress between the 3 cell lines.	

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
	HMLER-5 Normal human mammary epithelial cells HMECs as controls			Genes involved in cancer progression were down-regulated. Tumorigenic cells relatively unaffected by oxidative stress versus non-transformed cells. Genes involved in DNA repair up-regulated by oxidative stress. GSTs and peroxiredoxin up-regulated by oxidative stress but down-regulated during cancer progression. SOD and GPx were up-regulated by oxidative stress and cancer progression.	
Kang et al.[106]	Human breast cancer cells MCF-7, MDA-MB-231, and MDA-MB-435s Untreated cancer cells as controls	0, 15, or 25 μM EPA or DHA	Cell viability DNA synthesis Cell death via flow cytometry Apoptosis via TUNEL staining Intracellular ROS concentrations Caspase 8 activity	IC_{50} of DHA and EPA 20.2 and 57.4 μM, respectively, in MCF-7 cells. MDA-MB-231 and MDA-MB-435s less sensitive to EPA and DHA. DHA treatment for 72 h resulted in decreased DNA synthesis, decreased cell viability of MCF-7 cells and increased apoptosis. Knockdown of caspase 8 eliminated effect of DHA on cell death. Vitamins E and C and *N*-acetyl cysteine protected MCF-7 cells from cell death due to DHA. DHA treatment increased ROS production in cells.	

Abbreviations:
13-HPODE = linoleic acid hydroperoxide
DODE = 9,12-dioxo-10(*E*)-dodecenoic acid

gamma linolenic acid (GLA) and AA, both of which are n-6, and concluded that GLA and AA treatment resulted in the highest concentration of TBARS, whereas DHA yielded the lowest increase in TBARS. In contrast, Maheo et al.[100], using different cell lines, were able to increase TBARS four-fold with DHA supplementation. These differences are likely due to the use of different cell lines and concentrations of DHA. DHA treatment was also shown to decrease cell viability and DNA synthesis and enhance apoptosis[106]. However, Maheo et al.[100] also demonstrated a significant increase in GSH with DHA treatment, which would increase the capacity for GSH-mediated detoxification of lipid peroxidation metabolites. Additionally, n-3 treatment reduced the levels of vitamin E present within the cells, and treatment with vitamin E negated the cytotoxic and oxidative effects of n-3 in a number of the studies[98, 102, 106]. In a study specifically addressing the effects of HNE on breast cancer cells, Cipak et al.[103] showed that exposure of cells to supraphysiological levels of HNE (35 or 50 μM) resulted in enhanced cytotoxicity and decreased viability. Cells treated with HNE under conditions of elevated oxidative stress had increased levels of HNE-protein adducts compared to cells treated with HNE under basal conditions. Slade et al.[105] measured the changes in the proteome of breast cancer cells treated with a LA hydroperoxide, which affected a total of 98 proteins, including many found in the nucleus. However, this work was primarily for method development, and it was not possible to draw meaningful conclusions regarding lipid peroxidation and breast cancer risk.

Cell culture studies are advantageous for exploring the specific mechanisms by which n-3 and lipid peroxidation may affect cancer promotion. In addition to promoting

apoptosis and protein binding, n-3 and lipid peroxidation metabolites can alter membrane fluidity and signal transduction. Menendez et al.[99] showed that the activity of fatty acid synthase (FAS), a protein frequently over-expressed in cancers, was significantly reduced by treatment of breast cancer cells with ALA, EPA, DHA, GLA, and AA, and this effect was blocked by the addition of vitamin E. In a follow-up study, the same authors showed that ALA significantly reduced *HER2* transcription and expression; *HER2* over-expression is associated with more aggressive breast cancers with poorer prognoses and decreased survival times[101]. In a study that did not address the effects of supplemental n-3, Rysman et al.[104] showed that the inhibition of enzymes involved in saturated fatty acid synthesis, FAS and acetyl CoA-carboxylase (ACC), increased lipid peroxidation as well as protein motility within the cellular membranes and ultimately promoted cancer cell death. These studies offer important information about the association between dietary n-3, lipid peroxidation, and breast cancer but cannot be directly applied to the association within human beings.

Animal model studies

Table 3 includes a summary of animal studies measuring dietary n-3, lipid peroxidation, and breast cancer risk. In a seminal study, Gonzalez et al.[107] measured the effects of various ratios of corn oil and fish oil in the diets (20% energy from fat) of mice xenografted with human breast cancer cells. The mammary tumor TBARS significantly increased with increasing amounts of fish oil in the diets while mean tumor volume significantly decreased. Supplementation with vitamin E and TBHQ, a synthetic

Table 3. Summary of animal model studies examining lipid peroxidation and breast cancer.

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
Gonzalez et al.[107]	Female athymic nude mice implanted with human breast cancer cells MDA-MB-231 Corn oil diet fed mice served as controls	Corn and/or fish oil diet beginning 7-10 d following xenograft: ▪ 20% corn oil ▪ 15% corn oil, 5% fish oil ▪ 10% corn oil, 10% fish oil ▪ 5% corn oil, 15% fish oil ▪ 1% corn oil, 19% fish oil ▪ 1% corn oil, 19% fish oil + 2000 IU/kg vitamin E and 2% total fat TBHQ	Tumor incidence Mean tumor volume Breast carcinoma TBARS	TBARS sig. increased with increasing proportion of fish oil, while mean tumor volume decreased. Increase in TBARS was prevented by supplemental antioxidants, but mean tumor volume was not sig. lower than high corn oil diets in this group. Increased time that fish oil diet remained in food jar also sig. increased tumor TBARS.	
Hardman et al.[86]	Female athymic nude mice implanted with human breast cancer cells MDA-MB-231	Corn and/or fish oil diet beginning 3 wks following xenograft: ▪ 5% corn oil ▪ 5% corn oil + 2000 IU/kg vitamin E ▪ 3% fish oil, 2% corn oil ▪ 3% fish oil, 2% corn oil + 2000 IU/kg vitamin E 5 mg/kg body weight every 4 days doxorubicin in 5 mice per group for 5 weeks	Mean tumor volume Tumor, liver, and colon TBARS Fatty acid composition in mitochondria and microsomes of colon, liver, and tumor tissues Catalase, SOD, and GPx activity in tissues	GPx activity sig. decreased in tumor tissue of mice consuming the fish oil diet + vitamin E. There were no other sig. differences in antioxidant enzyme activities between diets or tissues. Vitamin E with fish oil diet sig. decreased tumor TBARS, although tumor TBARS of fish oil diet were not sig. different than corn oil. Vitamin E with corn oil diet prevented increased tumor TBARS with doxorubicin but not in fish oil diet. Fish oil diet sig. increased liver TBARS, which was suppressed with vitamin E with or without doxorubicin. Fish oil diets with doxorubicin sig. halted tumor growth compared to corn oil diets.	GPx reported to be more important than catalase in neutralizing lipid hydroperoxides.

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
				GPx activity accounted for 78% of increased TBARS following doxorubicin for 19% of variation in tumor growth.	
Hilakivi-Clarke et al.[108]	Rat model of mammary cancer induction with DMBA Low-fat 17:1 diet as control	Rat pups fed low-fat (16% energy) or high-fat (39% energy) diets between postnatal days 2 and 5: • 2:1 n-6:n-3 • 17:1 n-6:n-3	Tumor incidence Tumor latency Mammary gland morphology Apoptosis via TUNEL Cell proliferation *COX-2* expression PPARγ activity *Cyclin D1* expression Lipid hydroperoxide concentration *BRCA1* and *Caveolin-1* expression 8-OHdG	Low-fat 2:1 diet reduced the incidence and multiplicity of mammary tumors, whereas high-fat 2:1 diet increased the incidence and multiplicity of tumors. No differences in tumor incidence and multiplicity between low- and high-fat 17:1 diets. Increased mammary gland differentiation in both 2:1 diets. High-fat 2:1 diet had highest level of lipid hydroperoxides, while low-fat 17:1 diet had lowest. Low-fat 2:1 diet showed modest increase in lipid hydroperoxides. Both n-3 diets showed decreased *Cox-2* expression. Low-fat n-3 diet showed increased and decreased proliferation. PPARγ activity lowest and cyclin D1 expression highest in high-fat 2:1 diet. PPARγ activity sig. increased in low-fat 2:1 diet. *BRCA1* expression highest in n-3 groups. *Caveolin-1* expression highest in n-3 groups. 8-OHdG increased in high-fat 2:1 diet but decreased in low-fat 2:1 diet.	Supplement article
El-Mesery et al.[109]	Female Swiss albino mice for tumor experiments via injection with	0, 125, or 250 mg/kg DHA for 20 d 0, 125, or 250 mg/kg DHA following cisplatin injection	Mean tumor size TBARS Serum C-reactive protein concentration Leukocyte count	High-dose DHA sig. reduced mammary tumor size and serum TBARS in mouse model. There was no correlation between TBARS and tumor size. High-dose DHA + cisplatin for 10 d sig.	

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
	Ehrlich ascites carcinoma cells (mammary origin) No injection for control mice Male Sprague-Dawley rats for nephrotoxicity experiments with cisplatin injection No injection for control rats		Kidney glomerular function TNF-α and GSH concentrations	decreased kidney TBARS and TNF-α and increased kidney GSH in rat model.	
Kang et al.[106]	Female athymic nude mice implanted with human breast cancer cells MCF-7 or MDA-MB-435s Control diet (5% corn oil) mice served as controls	Control diet + 5% fish oil	Mean tumor size Plasma and breast, uterus, skin, and tumor fatty acid concentrations Morphological characteristics Cell proliferation and apoptosis via immunohistochemistry	Fish oil diet sig. decreased tumor volume and cell proliferation and increased apoptosis in MCF-7-implanted mice.	
Schubert et al.[110]	ATM-deficient mice (at high risk for multiple cancers, including mammary) Control diet fed mice served as controls Wild-type mouse splenocytes used as controls in *in vitro* analyses	EPA-enriched fat blend diet AA-enriched fat blend diet	Plasma and erythrocyte membrane EPA 8-OHdG concentration	No differences between diets in latency to tumor development. EPA treatment sig. increased 8-OHdG compared to control and AA treated animals.	

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
Kansal et al.[111]	Female Wistar rats fed DMBA to induce mammary tumor formation 1.0 mL olive oil fed animals with or without DMBA treatment as controls	• 0.5 mL fish oil • 0.25 mL fish oil • 20 mg/kg body weight celecoxib • 30 mg/kg body weight celecoxib • 0.5 mL fish oil + 20 mg/kg celecoxib • 0.25 mL fish oil + 30 mg/kg celecoxib	DNA fragmentation Mammary tissue and liver mitochondria TBARS SOD and catalase activities GSH concentration	Fish oil with or without celecoxib sig. increased DNA fragmentation. Carcinogen treatment sig. increased TBARS. Fish oil treatment sig. increased liver TBARS but decreased or did not change mammary TBARS. Celecoxib + fish oil sig. decrease liver TBARS to control levels. Sig. decrease in SOD and catalase activities and GSH concentrations in carcinogen-treated animals. Fish oil + celecoxib sig. increased liver and mammary SOD and catalase activity (30 mg/kg celecoxib group) compared to carcinogen controls. Fish oil + celecoxib sig. increased mammary and liver GSH compared to carcinogen controls. 0.5 ml fish oil group also had sig. increased mammary GSH.	
Manni et al.[112]	Female Sprague Dawley rats injected with N-methyl-N-nitrosourea (MNU) to induce mammary cancer Saline-injected rats or 20% corn oil diet as control	• 20% corn oil • 20% corn oil + 100 μg/kg tamoxifen • 17% fish oil + 3% corn oil • 17% fish oil + 3% corn oil + 100 μg/kg tamoxifen • 10% fish oil + 10% corn oil • 10% fish oil + 10% corn oil + 100 μg/kg tamoxifen	Cell proliferation and apoptosis via immunohistochemistry Mammary tissue TBARS, GPx activity, GSH levels, and 8-OH-dG Plasma protein carbonyls via DNPH Mammary tissue 8-isoprostane via ELISA Plasma free and protein-bound GSH Plasma and mammary tissue fatty acid distribution	No effect of fish oil or tamoxifen on preneoplastic lesions. 17% fish oil diet sig. decreased cell proliferation but no effect on apoptosis. No sig. effects of interventions on mammary TBARS or plasma protein carbonyls. Both fish oil diets sig. decreased 8-isoprostane levels in the mammary tissue. 10% fish oil diets sig. increased GPx activity, while 17% fish oil GPx activity did not differ from controls. GSH levels sig. lower in 10% fish oil group and borderline sig. lower in 17% fish oil group.	Authors suggest that fish oil and tamoxifen block progression of hyperplasia to carcinoma. Reduction of 8-isoprostane likely due to lower amounts of arachidonic acid.

Abbreviations:
DMBA = 7,12-dimethylbenz[a]anthracene
TNF-α = tumor necrosis factor-α
8-OHdG = 8-hydroxy-2'-deoxyguanosine
13-HODE = 13-hydroxyoctadecadienoic acid
ATM = gene product mutated in ataxia telangiectasia
MNU = N-methyl-N-nitrosourea

antioxidant, reduced the concentration of TBARS but also increased the size of mammary tumors to the levels seen in the high corn oil diet, demonstrating that the chemopreventive effect of fish oil was due to lipid peroxidation. A subsequent study conducted in the same system by Hardman et al.[86] explored corn and fish oils as part of a low-fat (5% energy from fat) diet. The authors were unable to demonstrate a significant difference in mammary tumor TBARS between the corn and fish oil groups, although the supplementation of the fish oil diet with vitamin E significantly reduced tumor TBARS. The fish oil diet significantly increased liver TBARS compared to the corn oil diets; again, this effect was suppressed by vitamin E. Treatment with doxorubicin, an anti-cancer drug, in addition to the fish oil diet significantly decreased tumor growth compared to the corn oil diet. The authors also measured the activity of the antioxidant enzymes SOD, catalase, and GPx in the colon, liver, and tumor tissues, which did not significantly differ between treatments with the exception of a significant decrease in GPx activity in the tumors of mice consuming the fish oil diet with vitamin E. Using linear regression modeling, the authors concluded that the activity of GPx accounted for 78% of the variation in tumor size, while increased TBARS following drug treatment accounted for a further 19%.

The differences in a low- versus high-fat diet with or without supplemental levels of n-3 was further explored by Hilakivi-Clarke et al.[108]. In this study, rats were fed a low-fat (16% energy from fat) or high-fat (39% energy from fat) diet with a ratio of n-6:n-3 of either 2:1 or 17:1 beginning between two and five days following birth; mammary tumors were induced via 7,12-dimethylbenz[a]anthracene (DMBA). There

were no differences in mammary tumor incidence and multiplicity between the two 17:1 diets, but the low-fat 2:1 diet decreased and high-fat 2:1 diet increased the development of mammary tumors. The concentration of lipid hydroperoxides was highest in the high-fat 2:1 diet and modestly increased in the low-fat 2:1 diet; the lowest concentrations of lipid hydroperoxides were evident in the low-fat 17:1 diet. The concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) followed similar patterns to the lipid hydroperoxides, with increased levels in the high-fat 2:1 diet and decreased levels in the low-fat 2:1 diet. This study also measured the effects of the diets on gene transcription and expression; the tumor suppressors *BRCA1* and *caveolin-1* were highest while *Cox-2* was lowest in the n-3 groups. These results appear to implicate the importance of both absolute and relative amounts of fatty acids in the development of breast cancer. Although the gene expression profile did not favor cancer progression in both n-3 groups, the high-fat 2:1 group exhibited increased oxidative DNA damage, lipid hydroperoxides, and tumor incidence, suggesting that the detrimental effects of lipid peroxidation may outweigh the beneficial effects of n-3 on gene transcription when breast cancer is induced.

El-Mesery et al.[109] evaluated the effects of DHA in a mouse model of mammary cancer induced by Ehrlich ascites carcinoma cells. In contrast to the previously mentioned studies, 250 mg/kg body weight of DHA significantly decreased serum TBARS as well as mammary tumor volume. The effects of DHA and the cancer drug cisplatin were also addressed in a rat model; when 250 mg/kg body weight of DHA in addition to cisplatin were given to the animals for ten days, there was a significant

decrease in kidney TBARS and increase in kidney GSH. This suggests that treatment with DHA at this concentration for ten days is not toxic to the kidney.

Kansal et al.[111] addressed the effects of fish oil with or without celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, in a rat model of mammary carcinoma induced by DMBA. Although fish oil treatment significantly increased DNA fragmentation, it did not increase mammary TBARS. However, fish oil treatment did significantly increase liver TBARS, which were repressed to control levels with the addition of celecoxib. The fish oil and celecoxib treatment also significantly increased liver and mammary tissue SOD and catalase activity and GSH concentration. These results suggest that the use of a COX-2 inhibitor in addition to fish oil can enhance antioxidant defense and detoxification by GSH and suppress liver TBARS but still results in DNA damage.

Recently, Manni et al.[112] measured the effects of a 10% or 17% fish oil diet with or without tamoxifen, an anti-estrogen drug, in a rat model of breast cancer. The study did not address frank mammary tumor development but rather the formation of precancerous lesions, such as mammary hyperplasia. There were no significant differences in the development of such lesions between the treatments tested. However, the fish oil diets significantly decreased a marker of cell proliferation, suggesting a lower risk of cancer. The authors also measured markers of lipid peroxidation including mammary tissue TBARS and protein carbonyl levels in the plasma but found no significant effects of fish oil. The 10%—but not 17%—fish oil diet significantly increased the activity of GPx within the mammary tissue, indicating such a diet may up-regulate detoxification of lipid hydroperoxides.

In a mouse model implanted with MCF-7 human breast cancer cells, Kang et al.[106] demonstrated that a 5% fish oil diet significantly decreased mammary tumor volume and cell proliferation while increasing apoptosis; lipid peroxidation was not measured. Schubert et al.[110] assessed an EPA- or AA-rich diet on the development of several cancers, including mammary cancer, in a mouse model deficient for gene product mutated in ataxia telangiectasia (ATM). There were no differences in tumor latency, but EPA supplementation significantly increased 8-OHdG compared to AA supplementation. Although these two studies did not directly measure lipid peroxidation, each showed no effect or a reduced risk for breast cancer with n-3 supplementation. Again, n-3 supplementation increased the levels of oxidative DNA damage within the latter animal model.

Together, these animal research studies show that while diets supplemented with fish oil or long-chain PUFA including DHA increase markers of lipid peroxidation (usually TBARS), such diets are associated with a reduction in the incidence and size of mammary tumors. Although the oxidative stress induced by a diet high in PUFA increases the amount of oxidative DNA damage, an up-regulation of apoptosis and decrease in cell proliferation may limit the development and spread of tumors. Additionally, the up-regulation of certain antioxidant defense systems may also serve to reduce the risk of breast cancer.

Human studies

Table 4 summarizes notable work in observational (cross-sectional and case-control) human studies exploring the relationship between lipid peroxidation and breast cancer risk in women. Although a chief limitation of such studies is the inclusion of subjects with established disease processes that precludes the assignation of cause and effect, the studies offer information involving the potential role of lipid peroxidation in breast carcinogenesis and cancer progression in the general population.

Several studies examined the extent of oxidative stress in breast cancer cases compared to controls. Ray et al.[113] detected significantly higher levels of superoxide and hydrogen peroxide in the serum of breast cancer cases compared to non-cancer surgical patients as controls. Similar results were seen in a study conducted by Yeh et al.[114], wherein breast cancer patients had significantly higher superoxide levels than healthy controls. Chandramathi et al.[115] measured increased advanced oxidative end product in the urine of breast cancer cases compared to healthy controls, but the authors did not detect a significant difference in urinary hydrogen peroxide in cases versus controls.

Most studies that measured lipid peroxidation via TBARS in breast cancer cases and healthy, typically age-matched controls indeed demonstrated increased lipid peroxidation in cases over the controls[113, 114, 116-123]. One study conducted by Gerber et al.[124] reported lower plasma TBARS among breast cancer cases compared to controls; however, in contrast to the other studies included in Table 4, this study used non-cancer hospital patients as controls rather than healthy members of the general

Table 4. Summary of observational human studies examining lipid peroxidation and breast cancer in women.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Karihtala et al.[125]	Cross-sectional breast cancer patients n=80	8-OHdG, HNE, nitrotyrosine, DNA topoisomerase II binding protein I, and mismatch repair proteins 2 and 6 expression via immunohistochemistry of tumor tissues	HNE present in 86.8% of stage I tissues and usually co-expressed with nitrotyrosine. 8-OHdG expressed in 58.9% of tissues, and DNA repair proteins expressed in 55.9-65% of tissues. Stage I tumors had increased nitrotyrosine expression compared to hyperplastic tissues. Topoisomerase binding protein I expression was sig. associated with 8-OHdG expression in stage I tissues. 11 of 12 tissues negative for 8-OHdG in hyperplasia.	Nitrotyrosine is a marker of nitrogen stress. Stage I breast tumors 2 cm or less in greatest dimension and no spread to lymph nodes.
Carneiro et al.[116]	Cross-sectional breast cancer patients, n=59 Healthy female blood donors, n=76	CXCR4 expression in peripheral blood cells via PCR Plasma MDA via HPLC	Plasma MDA sig. higher in cancer patients versus controls. Stages II and III had sig. higher plasma MDA versus controls. No sig. differences in CXCR4 expression.	Numbers of samples in stages I and IV were low.
Vinothini and Nagini[126]	Cross-sectional breast cancer patients n=60 Adjacent normal tissues used as controls	Tissue cytochrome p450 and b5 content and ethoxyresorufin *O*-decarboxylase (EROD), methoxyresorufin *O*-decarboxylase (MROD), pentoxyresorufin *O*-decarboxylase (PROD), GST, and quinone reductase activity via spectrophotometry CYP1B1, CYP1A1, HNE, 8-OHdG, and anti-hexanoyl	Activities of phase I enzymes sig. increased in all cancer tissues compared to control tissues; the highest levels of activity were among grade III cancer tissues. The expression of CYP1A1 and 1B1 were sig. higher in breast cancer tissues and the highest in grade III tissues. GST and quinone reductase activities were sig. increased in all breast cancer tissues and were highest among grade I tissues. HNE, 8-OHdG, and HEL levels were sig. highest among breast cancer tissues compared to controls, with the highest levels among grade III tumors.	CYP1B1 catalyzes the formation of 4-OH-E_2 in mammary tissues, promoting carcinogenesis and oxidative stress. Increased markers of oxidative stress and phase I and II enzymatic activity in higher grades implicates role of lipid peroxidation in cancer promotion and progression. NF-κB expression can be

Reference	Study Design	Outcome Measurement(s)	Results	Notes
		lysine (HEL) expression via immunohistochemistry NF-κB, IκB, p-IκB, and IKKβ expression via immunohistochemistry and Western blotting	NF-κB, p-IκB, and IKKβ expression sig. up-regulated while IκB (inhibitor of NF-κB) sig. down-regulated in cancer tissues, particularly in grade III tissues. Overall changes were more pronounced in premenopausal tissues compared to postmenopausal.	up-regulated by oxidative stress and promote proliferation.
Karihtala et al.[127]	Cross-sectional breast cancer patients n=219	8-OHdG and HNE expression via immunohistochemistry of tumor tissues	8-OHdG expression in invasive carcinoma sig. lower than in hyperplasia (usual or abnormal) and ductal carcinoma *in situ*. There was a trend for increased HNE content in higher-grade ductal carcinoma *in situ* lesions. HNE expression was sig. highest in invasive carcinomas. Negative 8-OHdG and positive HNE sig. reflected different patient cohorts (aggressive vs. less aggressive). 8-OHdG staining sig. or borderline sig. correlated with estrogen receptor presence, low cell proliferation, and small tumors.	No associations between HNE and prognostic factors, and authors conclude this indicates a role in carcinogenesis. The authors also note it is impossible to determine if HNE levels are causes or consequences in breast carcinogenesis due to the study design. Authors conclude that low 8-OHdG levels in invasive cancers due to up-regulation of DNA repair during oxidative stress.
Gerber et al.[124]	Case-control Breast cancer cases, n=120 Hospital-based non-cancer controls, n=109	Questionnaire including diet history Plasma vitamin E Plasma lipids Plasma TBARS (in 95 cases and 70 controls)	Plasma vitamin E sig. higher in cases vs. controls. Significance remained after adjustment for total cholesterol in premenopausal patients only. Plasma total cholesterol sig. higher in cases compared to controls. Plasma TBARS sig. lower in patients compared to controls with adjustment for menopausal status.	
Kumar et al.[117]	Case-control Breast cancer cases, n=43 Age-matched	GPx, SOD, GST, and catalase activities Serum vitamins C and E, ceruloplasmin, and Se	Serum lipid peroxides and ceruloplasmin sig. increased in cases vs. controls. Cerulosplasmin was sig. higher in malignant vs. benign cancer cases.	Authors did not include their definitions of benign and malignant cancers.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	controls, n=20	Erythrocyte and serum lipid peroxide levels, likely via TBARS (no method described)	Serum vitamins C and E and Se were sig. decreased in cancer vs. controls. Vitamin C levels were sig. lower in malignant vs. benign cancer cases. Erythrocyte lipid peroxides were sig. higher in cancer cases vs. controls. Lipid peroxides were sig. higher in malignant vs. benign cancer cases. Catalase, GPx, SOD, and GST activities were sig. lower in cases vs. controls. Catalase and GPx activities were sig. lower in malignant vs. benign cancers.	
Huang et al.[118]	Case-control Breast cancer cases, n=35 Healthy controls, n=35	Serum MDA via HPLC Serum mineral content (Cu, Zn, Fe, and Se)	Breast cancer cases had sig. higher serum MDA, Cu, and Cu/Zn ratio compared to controls. Serum Se was sig. lower among stage III cases vs. controls. MDA and Cu levels highest among stages I and II.	
Ray et al.[113]	Case-control Breast cancer cases, n=54 Surgical, non-cancer controls, n=42	Serum superoxide and hydrogen peroxide productive via spectrophotometry Serum TBARS SOD, GPx, and catalase activities	Cases had sig. increased superoxide and hydrogen peroxide production and SOD and GPx activities compared to controls. The highest superoxide value was in stage II cases, and the highest hydrogen peroxide value was in stage III cases. SOD activity was highest in stage II, while GPx was highest in stage III. Cases had sig. decreased catalase activity compared to controls. The largest decrease was among stage II cases. Breast cancer cases, except stage IV, had sig. higher TBARS than controls. The highest TBARS values were in stage II cancer patients. There were differences in the results with respect to menopausal status. Postmenopausal patients had higher hydrogen peroxide, TBARS, and GPx increases and a greater decrease in catalase activity, while premenopausal patients had	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			increased superoxide and SOD activity.	
Gönenç et al.[128]	Case-control Breast cancer cases, n=26 Healthy controls, n=41	Plasma MDA via HPLC	Plasma MDA sig. higher in breast cancer cases compared to controls.	Study found similar results with lung cancer cases.
Yeh et al.[114]	Case-control Breast cancer cases, n=117 Healthy controls, n=117	Whole blood GPx, SOD, and GSH reductase activities Plasma TBARS Plasma vitamins A, C, and E Superoxide generation via lucigenin-based chemiluminescence Plasma GSH concentration	Plasma MDA and superoxide levels were sig. higher in cases compared to controls. SOD, GPx, and GSH reductase activities were sig. higher in cases vs. controls. Cases had sig. lower plasma GSH compared to controls. Plasma vitamin C was sig. lower in cases compared to controls. Superoxide radical levels were highest among estrogen receptor-negative, progesterone receptor-positive cases compared to double-positive cases. SOD activity was sig. lower among *HER-2/neu*-negative cases compared to positive cases.	
Şener et al.[119]	Case-control Breast cancer cases, n=56 Healthy controls, n=18	Serum total antioxidant capacity via ABTS radical cation decolorization assay Serum TBARS and lipid hydroperoxides via spectrophotometry Serum lipids	Serum total antioxidant capacity was sig. lower and serum TBARS were sig. higher in cases compared to controls. There were no sig differences in lipid hydroperoxides between cases and controls or within stages. TBARS were sig. highest among stage II cases, and total antioxidant capacity was sig. lowest among stages II and III.	
Kasapovic et al.[129]	Case-controls Breast cancer cases, n=53 Healthy controls, n=67	SOD, GPx, and GSH reductase activities. Plasma GSH concentration Plasma lipid hydroperoxides via spectrophotometry	CuZnSOD activity sig. lower in breast cancer cases of all ages compared to controls. GSH reductase and catalase activities sig. lower in ages 45->58. GSH concentration sig. lower among cases >58 vs. controls. Lipid hydroperoxides sig. higher among cases ages	Tumor cells may produce increased levels of hydrogen peroxide.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			45->58 vs. controls. CuZnSOD and catalase activities decreased with age among controls and cases.	
Suzana et al.[120]	Case-control Breast cancer cases, n=57 Healthy controls, n=139	Questionnaire including dietary history Plasma TBARS Plasma vitamins A and E via HPLC Toenail and hair Se content via ICP-MS	Controls had sig. lower plasma TBARS compared to cases. Controls reported sig. higher vitamin A and E intakes and had sig. higher plasma vitamin A vs. cases. Controls also had sig. higher toenail (but not hair) Se compared to cases. With binary logistic regression, 75th% quartile TBARS sig. increased OR for breast cancer. 25th% quartile or higher for biochemical marker of vitamins A and E also increased OR for breast cancer.	Authors used independent sample t-tests to compare cases vs. controls.
Rajneesh et al.[121]	Case-control Breast cancer cases, n=40 Healthy controls	Plasma lipid hydroperoxides and conjugated dienes Plasma TBARS Plasma GSH concentrations and GST, SOD, catalase, and GPx activities	Plasma TBARS sig. higher in cases vs. controls. Plasma conjugated dienes and lipid hydroperoxides also sig. higher in cases vs. controls. Plasma GSH concentrations and SOD, catalase, GST, and GPx activities sig. higher in cases vs. controls.	Stage I and II cancer patients only. No indication of number of controls included.
Chandramathi et al.[115]	Case-control Breast cancer cases, n=101 Healthy controls, n=95	First morning urinary advanced oxidative end product, hydrogen peroxide, MDA (via 1,1,3,3,-tetraethoxypropane), and ferric-reducing antioxidant power	There were no sig. differences in urinary MDA or hydrogen peroxide in breast cancer cases vs. controls (colorectal cancer cases were sig. higher in both than controls). Urinary advance oxidative end product was sig. increased, and ferric-reducing antioxidant power was sig. decreased in breast cancer cases vs. controls. There were no sig. differences between stages of breast cancer for the outcome measurements.	Study also evaluated colorectal cancer cases, n=49. Spot urine samples collected prior to any heavy meal.
Goswami et al.[122]	Case-control Breast cancer cases, n=60 Healthy	Whole blood GPx and SOD levels Plasma TBARS and conjugated dienes	TBARS, conjugated dienes, ferritin, and IL-6 sig. higher in cases vs. controls. GSH, GPx, and SOD levels and catalase activity sig. lower in cases vs. controls.	IL-6 and ferritin both considered inflammatory markers.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	controls, n=60	Plasma catalase activity Plasma IL-6 and ferritin concentrations via ELISA	Sig. positive correlation between conjugated dienes and IL-6 and stage of breast cancer. Sig. neg. correlation between SOD levels and stage of breast cancer and conjugated dienes.	
Kasapovic et al.[130]	Case-control Breast cancer cases, n=58 Healthy controls, n=60	Plasma lipid hydroperoxides Plasma catalase, GPx, and GSH reductase activities and GSH levels Plasma CuZnSOD activity	Plasma lipid hydroperoxides sig. increased in cases vs. controls and sig. increased in cases following chemotherapy in all age groups. CuZnSOD activity sig. decreased following chemotherapy in both age groups and in cases vs. controls among >58 y. GSH and GSH reductase sig. decreased among cases following chemotherapy, while GSH was sig. higher among cases vs. controls 45-58 y. Following radiation treatment, CuZnSOD levels sig. increased in both age groups compared to cases before treatment.	Stage II and III breast cancer cases only, undergoing chemotherapy or radiation treatment. Fasting blood samples taken before and after treatment. Authors used independent (cases vs. controls) and paired t-tests (before and after treatment).
Kedzierska et al.[123]	Case-control Invasive breast cancer cases, n=35 Benign breast disease cases, n=24 Health controls, n=40	Urinary 8-isoPGF$_{2\alpha}$ Plasma TBARS	Invasive breast cancer cases had sig. higher urinary 8-isoPGF$_{2\alpha}$ compared to controls and women with benign breast disease. Plasma TBARS were also sig. higher in women with invasive breast cancer compared to controls and women with benign breast disease.	

Abbreviations:
CXCR4 = chemokine (C-X-C motif) receptor 4
EROD = ethoxyresorufin *O*-decarboxylase
MROD = methoxyresorufin *O*-decarboxylase
PROD = pentoxyresorufin *O*-decarboxylase
HEL = hexanoyl lysine
ABTS = 2,2'-azino-bis(3-ethylbenz-thazoline-6-sulphonic acid) diammonium salt
8-isoPGF$_{2\alpha}$ = 8-isoprostaglandin F$_2$

population. Because the controls were also undergoing treatment for disease, it is possible that the controls' levels of lipid peroxidation were elevated due to underlying disease or illness.

Many studies also utilized more specific measurements of lipid peroxidation than TBARS. In a cross-sectional study of 80 breast cancer patients, Karihtala et al.[125] used immunohistochemical staining of tumor tissues to measure the expression of HNE, 8-OHdG, nitrotyrosine, and several markers of DNA repair. The authors detected the presence of HNE in 86.5% of stage I breast tumors, defined as less than two cm at greatest dimension and without lymph node involvement, and HNE was usually co-expressed with nitrotyrosine, a marker of nitrosative damage to proteins. 8-OHdG was expressed at lower levels in stage I tumor tissues (58.9%) and shared similar expression patterns with DNA repair proteins (55.9-65%). In a subsequent study, the authors again demonstrated lower 8-OHdG formation in invasive breast cancer tissues than in hyperplasia or ductal carcinoma *in situ*[127]. HNE formation was greatest in the invasive cancer tissues, and individuals with HNE but not 8-OHdG expression comprised those subjects with the most aggressive breast cancers. The results from these two studies suggest that the expression of 8-OHdG and DNA repair proteins are closely related, which would account for repair of DNA damaged due to oxidative stress. On the other hand, HNE is associated with frank tumor development and cancer progression.

In another immunohistochemical study, Vinothini and Nagini[126] detected significantly increased levels of HNE, 8-OHdG, and anti-hexanoyl lysine in breast tumor tissues compared to adjacent normal tissues. The highest levels of these markers of

oxidative stress were seen among grade III breast cancer tissues, which are defined histologically to have a high risk of recurrence[131]. The authors also measured increased activities and expression of phase I enzymes (such as CYP) in breast cancer tissues, particularly grade III tissues. A similar trend was seen with expression of the NF-κB family of transcription factors and regulatory proteins, which are involved in controlling cell proliferation. The activity of GST was significantly up-regulated in breast cancer tissues, with the highest activity in grade I tissues. The results of this study indicate that markers of lipid peroxidation are increased with the increasing grade of breast cancer, suggesting the participation of lipid peroxidation in cancer progression and risk of recurrence. Additionally, the up-regulation of antioxidant enzymes in grade I (low risk of recurrence) and phase I detoxification enzymes in grade III demonstrate an attempt at homeostasis due to elevated oxidative stress within the tumor tissues. The increased expression of NF-κB family members demonstrate that transcriptional regulation is also involved in cancer progression.

Huang et al.[118] specifically measured serum MDA via HPLC in breast cancer patients and healthy controls. The cancer cases had significantly higher serum MDA relative to controls, and the highest levels of MDA were present among stages I and II, which include tumors that are less than five cm at the widest dimension and may or may not have spread to an axillary lymph node[132]. Gönenç et al.[128] also measured MDA in the plasma of breast cancer cases and healthy controls with HPLC and demonstrated significantly higher MDA in cases compared to controls. Lastly, Chandramathi et

al.[115] specifically measured urinary MDA in breast cancer cases and healthy controls, but there were no significant differences between cases and controls.

Several researchers measured circulating lipid hydroperoxides via the oxidation of ferrous ions and subsequent binding of ferric ions to xylenol orange, which can be measured spectrophometrically, in breast cancer patients and healthy controls. Şener et al.[119] did not detect any differences in serum lipid hydroperoxides between breast cancer cases and controls. However, Kasapovic et al.[62] demonstrated increased plasma lipid hydroperoxides in breast cancer patients between the ages of 45 and 58 years old compared to age-matched controls. Rajneesh et al.[121] measured plasma conjugated dienes in addition to lipid hydroperoxides and found both markers significantly elevated in stage I and II breast cancer cases compared to controls. In an additional study conducted by Kasapovic et al.[130], plasma lipid hydroperoxides were significantly higher in stage II and III breast cancer cases than controls and were further increased following chemotherapy. Goswami et al.[122] also measured plasma conjugated dienes, the first step of free radical-induced peroxidation of PUFA, which were found to be significantly higher in breast cancer cases compared to controls. Additionally, the level of conjugated dienes significantly and positively correlated with the stage of breast cancer within this population. Kedzierska et al.[123] measured urinary 8-isoprostaglandin F_2 as a marker of lipid peroxidation (of n-6) and detected significantly higher levels in invasive breast cancer cases compared to healthy controls or women with benign breast disease. Altogether, the results of these specific measurements of lipid peroxidation in observational human studies indicate that lipid peroxidation is present at

higher levels in individuals with breast cancer than in healthy individuals and that increased lipid peroxidation is positively associated with breast cancer progression and severity.

A number of the studies described in Table 4 also include measurements of antioxidant enzyme activity and dietary antioxidant status in breast cancer cases and healthy controls. Several studies demonstrate increased activity of SOD, GPx, GSH reductase, catalase, and GST in the blood of women with breast cancer compared to controls[113, 114, 121]. However, Kumar et al.[117] concluded that the activities of catalase, GPx, SOD, and GST were significantly lower in cases compared to controls. Kasapovic et al.[129] also demonstrated decreased SOD activity among breast cancer cases and lower GSH reductase and catalase activities among breast cancer cases between the ages of 45 and 58; additionally the authors measured significantly decreased SOD activity following chemotherapy but increased activity following radiation treatment among breast cancer patients[130]. Although some studies indicate increased antioxidant enzyme activity among breast cancer patients, it is not possible to draw conclusions given the body of evidence showing an opposite association. It is likely that the measurement of enzyme activities varies with regards to the methods employed as well as characteristics of the study subjects, including genetic variations, dietary intake, and medication use.

With regards to dietary antioxidant status, Gerber et al.[124] noted significantly higher plasma vitamin E in breast cancer cases compared to controls; again, this study used individuals currently under the care of a physician as controls, which may have

biased the results. Kumar et al.[117] measured significantly lower levels of vitamins C and E and selenium in breast cancer cases compared to age-matched controls. Significantly lower selenium status among breast cancer cases relative to controls was also demonstrated by Huang et al.[118]. Yeh et al.[114] detected decreased plasma vitamin C in breast cancer cases relative to controls, and Suzana et al.[120] measured significantly higher plasma vitamin A and toenail selenium in healthy controls compared to breast cancer patients. A decrease in antioxidant status among breast cancer patients is expected due to an increase in oxidative stress in addition to the presence of disease, and the results from these studies corroborate such an association, particularly with vitamin C and selenium. However, the diet consumed by each subject will also influence the circulating levels of antioxidant nutrients, and few studies included a subjective assessment of the typical diet consumed by subjects.

In addition to dietary antioxidants, several studies also measured the concentration of GSH within the blood of breast cancer patients and controls. Yeh et al.[114] measured significantly lower concentrations of plasma GSH in breast cancer cases compared to controls, while Kasapovic et al.[129] detected this difference only in breast cancer patients greater than 58 years old. On the other hand, a subsequent study by Kasapovic et al.[130] showed increased plasma GSH levels in cases between the ages of 45 and 58. Rajneesh et al.[121] concluded there was a significant increase in plasma GSH in breast cancer cases compared to controls. As with antioxidant enzyme activity, it is likely that GSH concentrations vary due to genetics, diet, and other factors, which may explain the conflicting results.

Table 5 summarizes two prospective cohort studies that measured the associations between lipid peroxidation and breast cancer risk among women from the general population. Prospective cohort studies and nested case-control analyses conducted within them are not affected by the same limitations as traditional case-control studies, which recruit subjects after the diagnosis of disease. The latter studies may be influenced by recall bias, a type of information bias whereby subjects with the disease recall their exposures prior to diagnosis differently than the control subjects. A distinct advantage of prospective cohort studies is the collection of exposure data, such as dietary and other environmental exposures, at the baseline of the study; subjects are then followed for several years until an adequate number have been diagnosed with the disease of interest to conduct statistical analyses. In addition to reducing recall bias, the collection of baseline variables allows for inference of cause and effect that is not possible with retrospective studies, such as traditional case-control studies. However, randomized controlled trials remain the gold standard for proving cause and effect.

In a nested case-control analysis within the Singapore Chinese Health Study, a large, population-based prospective cohort, Gago-Dominguez et al.[133] evaluated polymorphisms of the *GST* isoforms and marine n-3 intake assessed via food-frequency questionnaire in relationship to breast cancer risk. No results for marine n-3 were reported independently, but among postmenopausal women possessing two null alleles for *GSTT1* (conferring reduced activity), marine n-3 intake in the highest three quartiles significantly reduced the odds for breast cancer by 46% compared to those women in the

Table 5. Summary of prospective cohort studies examining lipid peroxidation and breast cancer risk.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Gago-Dominguez et al.[133]	Population-based prospective cohort, nested case-control analysis Breast cancer cases, n=258 Healthy controls, n=670	Baseline diet via food-frequency questionnaire Breast cancer diagnosis via national registry *GSTM1, T1,* and *P1* genotyping	No sig. associations between *M1* and *P1* genotype and breast cancer risk. *T1* null genotype sig. associated with reduced risk for breast cancer. Borderline sig. reduced risk for breast cancer among *GSTT1* null/null post-menopausal subjects with marine n-3 intake in the highest 3 quartiles. Sig. decrease risk in *GSTP1* heterozygous post-menopausal subjects in highest 3 quartiles of marine n-3 intake. Post-menopausal subjects with *GSTM1* or *GSTT1* null/null and *GSTP1* heterozygous had sig. decreased risk for developing breast cancer.	Authors interpret results as suggesting anti-carcinogenic effect of lipid peroxidation.
Lee et al.[134]	Population-based prospective cohort, nested case-control analysis Breast cancer cases, n=327 Healthy controls, n=654	Baseline questionnaire including dietary intake Breast cancer diagnosis via national registry Baseline urinary 1-hydroxypyrene, 2-naphthol, MDA, and 8-OHdG	No sig. differences between cases and controls for any of the outcome measurements.	Authors note that estrogen may inhibit lipid peroxidation.

lowest quartile of marine n-3 intake. *GSTP1* heterozygous postmenopausal women in the highest three quartiles of marine n-3 intake had 51% lower breast cancer odds compared to women in the lowest intake quartile. When these genotype characteristics were evaluated together, postmenopausal women with both of these beneficial genotypes and in the highest three quartiles of marine n-3 intake had 64% lower odds of developing breast cancer. Because the breast cancer risk reducing effects of high marine n-3 intake were seen only among postmenopausal women with lower GST activity, the authors concluded that the beneficial effects of n-3 on breast cancer risk may be largely due to increased lipid peroxidation, which would be suppressed in women with more active GST isoforms.

Lee et al.[134] conducted a nested case-control analysis of lipid peroxidation and breast cancer risk within the Shanghai Women's Health Study, another large population-based prospective cohort. Baseline urinary MDA and 8-OHdG were measured in addition to markers of polycyclic aromatic hydrocarbons, likely breast carcinogens to which humans may be exposed through cigarette smoke and air pollution. The authors found no associations between these baseline markers and breast cancer development in the cohort. Although subjective dietary data were not included in this experiment, the results suggest that lipid peroxidation and oxidative DNA damage are not causally associated with breast cancer among Chinese women in Shanghai.

Finally, human intervention studies addressing dietary n-3, lipid peroxidation, and breast cancer risk are summarized in Table 6. Unfortunately, no single intervention study has addressed these three areas simultaneously to date. Thangaraju et al.[135] examined

Table 6. Summary of human intervention studies examining lipid peroxidation and breast cancer.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
Thangaraju et al.[135]	Postmenopausal female breast cancer patients, n=64	10 mg tamoxifen twice daily for 6 months	Serum TBARS at 0, 3, and 6 months Serum retinol, ascorbic acid, vitamin E, GSH, ceruloplasmin, uric acid, and Se at 0, 3, and 6 months Plasma catalase, SOD, GPx, and GST activities at 0, 3, and 6 months	Tamoxifen treatment sig. decreased TBARS and 3 and 6 months. Treatment sig. increased serum antioxidants and increased activity for all enzymes measured.	No control group Tamoxifen activated by phase I enzymes contains phenolic hydroxyl group, which may account for antioxidant effects. Tamoxifen functions to suppress cell proliferation, not cause cell death.
Wu et al.[53]	Postmenopausal healthy female vegans and lacto-ovovegetarians, n=27	Single-blinded, placebo controlled study of: • 6 g corn oil/d • 6 g DHA-rich algae oil/d (2.14 g DHA/d)	Fasting plasma lipids Plasma copper-induced LDL oxidation and TBARS Plasma lipids and vitamin E concentrations Spot urine estrogen metabolites, F_2-isoprostane, and 8-iso-prostaglandin F_2	TBARS from oxidized LDL increased sig. in DHA supplemented group vs. corn oil supplemented. Total cholesterol sig. decreased with DHA supplementation. No sig. differences in vitamin E status, estrogen metabolites, or urinary prostaglandins between groups.	
Muralikrishnan et al.[81]	Postmenopausal female breast cancer patients, n=60 Healthy controls, n=15	5 treatment groups: • No treatment • Breast cancer patients not treated with tamoxifen • Breast cancer patients treated with	Plamsa TBARS Erythrocyte catalase, SOD, GPx, and GST activities	TBARS sig. increased in untreated breast cancer patients and patients receiving tamoxifen without vitamin C compared to controls. TBARS reverted to control levels in both groups treated with vitamin C. The same trend was seen with antioxidant enzyme activity, which sig. decreased	Authors do not report how much vitamin C was supplemented to patients.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
		tamoxifen • Breast cancer patients treated with vitamin C after 45 d of tamoxifen treatment • Breast cancer patients treated with vitamin C after 90 d of tamoxifen treatment		in breast cancer patients receiving no treatment or tamoxifen alone. Vitamin C supplementation restored enzyme activity to control levels.	

the effects of treatment with tamoxifen, an anti-estrogen drug commonly used to treat breast cancer, on serum markers of lipid peroxidation and antioxidant function. Although no control group was utilized, 10 mg tamoxifen twice daily significantly decreased fasting serum TBARS at three and six months compared to the values at baseline. The treatment significantly increased the serum concentrations of vitamin E, retinol, ascorbic acid, selenium, and GSH and significantly enhanced the activity of plasma catalase, SOD, GPx, and GST at three and six months. These results were not surprising given that tamoxifen can function as an antioxidant following activation by phase I enzymes in the body, and they suggest that suppression of lipid peroxidation may be one pathway by which tamoxifen effectively treats breast cancer.

Muralikrishnan et al.[81] evaluated tamoxifen treatment with or without supplemental vitamin C in postmenopausal breast cancer patients and a group of untreated controls. In contrast to the work of Thangaraju et al., these authors showed that tamoxifen treatment significantly increased plasma TBARS. The addition of vitamin C beginning at either day 45 or 90 of tamoxifen treatment reduced plasma TBARS to the levels of healthy controls without breast cancer. This study also showed that tamoxifen treatment significantly suppressed the activities of catalase, SOD, GPx, and GST, and this down-regulation was eliminated with the vitamin C supplementation. The authors do not report the quantity of vitamin C that was given to patients, but the results suggest that tamoxifen may increase lipid peroxidation and that the consumption of additional dietary antioxidants during tamoxifen treatment may be warranted in breast cancer patients. The authors also do not report the duration of the treatment and at what point blood samples

were collected, rendering it impossible to evaluate a potential timing effect. It is possible that the patients were not fasting prior to blood collection, and the contents of the most recent meal may have increased the levels of TBARS detected. Blood samples in Thangaraju et al.[135] were collected following a 12-24-hour fast; therefore, the effect of tamoxifen to decrease serum TBARS is not influenced by recent dietary intake in that study. More research is needed to clarify the effect of tamoxifen on lipid peroxidation as well as its interaction with dietary n-3 and antioxidants in so doing.

In a study of healthy vegetarian postmenopausal women, Wu et al.[53] randomly assigned subjects to consume a 6 g corn oil or 6 g DHA-rich algae supplement daily for six weeks. In blood samples collected following a 12-hour fast, women consuming the DHA supplement showed significantly increased LDL TBARS following induction of oxidation with copper versus women consuming the corn oil supplement. However, total plasma cholesterol was significantly reduced in the DHA group compared to the corn oil group, and there were no significant differences in plasma vitamin E, urinary estrogen metabolites, or urinary prostaglandins between the two groups. These results suggest that LDL from women consuming about 2.14 g DHA per day are more susceptible to lipid peroxidation because of the higher membrane content of DHA, but women consuming this level of DHA do not exhibit depletion of vitamin E and may lower their overall cholesterol. The results of the urinary estrogen metabolites suggest neither a protective nor enhancing effect of such a DHA supplement on breast cancer risk.

Although retrospective human data suggest increased lipid peroxidation in women with breast cancer compared to healthy controls, prospective cohort studies do not

support a causative role of lipid peroxidation of dietary n-3 in breast cancer development. In fact, some prospective data indicate a protective role of marine n-3 due to lipid peroxidation. More randomized controlled trials will be needed to parse out this association.

Conclusions

Public health recommendations to increase dietary consumption of n-3 and/or fatty fish for cardiovascular disease prevention are likely to expand due to the documented beneficial effects of these dietary components on plasma lipid profiles and cardiac events. However, the effects of such recommendations on *in vivo* lipid peroxidation and the resulting biological consequences remain poorly characterized. Of particular interest is the role of dietary n-3 and lipid peroxidation in reducing the risk or slowing the progression of human cancers, including breast cancer.

Lipid peroxidation of n-3 produces several reactive polar aldehydes that have been shown to bind DNA, alter enzyme functions, and trigger cell death. Antioxidants from the diet or present within the organism can slow or prevent the process of lipid peroxidation as well as detoxify the reactive products. In studies of cancer cells, n-3 and/or lipid peroxidation metabolites significantly altered signal transduction and gene expression and ultimately reduced cancer cell viability. However, it is not likely that such results are directly applicable to humans.

The results from animal studies addressing dietary n-3 and the risk of mammary cancer have been less conclusive. Although some demonstrated that n-3 decrease the size

and number of mammary tumors due to enhanced lipid peroxidation, differences in absolute and relative amounts of fatty acids consumed across the studies limit the consistency and generalizability of the results from these studies. In contrast, human case-control studies fairly consistently showed that women with breast cancer have increased lipid peroxidation relative to non-cancer controls. However, the observational studies were also inconclusive regarding alterations in antioxidant status associated with breast cancer, with studies indicating either increases or decreases in antioxidant concentrations or functions in women with breast cancer versus non-cancer controls.

Human cross-sectional studies that have utilized immunohistochemical staining of cancer and surrounding non-cancer tissues have suggested that lipid peroxidation is associated with breast cancer progression rather than carcinogenesis. This association is also evident from the few prospective cohort studies that have not demonstrated significantly increased risks for breast cancer with increased n-3 intake or *in vivo* lipid peroxidation.

To fully characterize the relationships between dietary n-3, *in vivo* lipid peroxidation, and breast cancer risk, additional prospective and intervention data will be needed. There are presently no intervention studies that directly assess these associations, and the limited data regarding the function of tamoxifen to increase or decrease lipid peroxidation in treating breast cancer are conflicting. With increasing access to highly sensitive techniques to ascertain antioxidant status and quantify in vivo lipid peroxidation, controlled studies of dietary n-3 supplementation will provide

valuable information regarding this intriguing and controversial topic of whether n-3 and lipid peroxidation are beneficial or detrimental to human health.

Chapter II: Moderate consumption of omega-3 (n-3) fatty acids significantly increases *in vivo* lipid peroxidation in healthy post-menopausal women

Introduction

Lipid peroxidation is an autocatalytic, free radical-induced process by which polyunsaturated fatty acids (PUFA) are degraded into lipid hydroperoxides and reactive lipophilic aldehydes and ketones. This process can occur in food products as well as in biological membranes, where the production of lipophilic aldehydes such as 4-hydroxy-2-*trans*-hexenal (HHE) and 4-hydroxy-2-*trans*-nonenal (HNE) may result in cellular damage or death[35, 136]. In addition to vitamin E deficiency and impaired endogenous antioxidant systems, a diet high in PUFA would be expected to increase total lipid peroxidation within an organism[98, 137, 138].

Fish oil is a rich source of the long chain omega-3 (n-3) PUFA eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), and many studies have linked fish oil consumption to reduced risk of cardiovascular disease, cognitive decline, and cancer[6, 139, 140]. The American Heart Association currently recommends that adults consume two servings of fatty fish per week (about 500 mg EPA + DHA per day) to reduce the risk of coronary heart disease[140]. However, increasing intake of fish oil would likely increase lipid peroxidation within the body, and as atherosclerosis is highly influenced by lipid peroxidation, the concomitant increase of PUFA due to fish consumption may increase the risk of atherosclerotic cardiovascular disease[39]. Increased consumption of the essential n-3 α-linolenic acid (ALA, 18:3 n-3), found in plant sources such as canola oil, flaxseeds, and soybeans, would also be associated with an increase in *in vivo* lipid peroxidation.

Studies examining fish oil consumption and cancer risk have been largely inconclusive[141]. With respect to cancer, lipid peroxidation products such as HNE have been shown to bind to amino acids and DNA, resulting in protein dysfunction and genetic mutations[35]. The process of lipid peroxidation and its reactive products have also been shown to result in cancer cell death and augment chemotherapy during cancer treatments[98, 142]. These disparate functions of lipid peroxidation suggest that it may initiate cancer development yet also eliminate cancer cells from the body.

Recently, this laboratory developed a method to identify and quantify polar and nonpolar aldehydic products of lipid peroxidation and validated this method in humans[143]. However, no research has been conducted on the response of such compounds to a well-controlled feeding study including fish oil and ALA. As a supplement to a crossover feeding study examining the influence of dietary fats on the risk of breast cancer, the following study was completed to compare the effects of a low-fat diet with or without n-3 PUFA on whole body lipid peroxidation in post-menopausal women. The results from this study suggest that a moderate increase in n-3 consumption increases *in vivo* lipid peroxidation, as measured by urinary lipophilic aldehydes and ketones measured as DNP-hydrazones.

Materials and Methods

Chemicals and supplies

Trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), hexanal (98%), pentan-2-one (97%), hept-2-enal (97%), hepta-2,4-dienal (90%), decanal, deca-2,4-dienal,

methanol, water, ethyl acetate, 1-chloro-2,4-dinitrobenzene, acetonitrile, N-acetyl-L-cysteine, and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO). Thiobarbituric acid, hydrochloric acid, methylene chloride, and dibasic phosphate were obtained from J.T. Baker (Philipsburg, NJ). Acetone, monobasic phosphate, and dimethyl sulfoxide were purchased from Fisher Scientific (Fair Lawn, NJ); hexane from EMD Chemicals (Gibbstown, NJ); malondialdehyde tetramethyl acetal from Kodak (Rochester, NY); DNPH-derivative of butyraldehyde from Supelco Analytical (Bellefonte, PA). DNPH-derivatives of butanone, octanal, non-2-enal, 4-hydroxyoct-2-enal, and 4-hydroxydec-2-enal were generously provided by Dr. Esterbauer, University of Graz (Graz, Austria). 4-hydroxy-2-*trans*-hexenal and 4-hydroxy-2-*trans*-nonenal were purchased from Cayman Chemicals (Ann Arbor, MI). *L*-ascorbic acid was procured from Eastman (Rochester, NY), and ammonium formate was purchased from Fluka (Buchs, Switzerland). All chemicals and solvents were HPLC-grade and filtered and degassed prior to use. Silica gel thin layer chromatography (TLC) plates (20 cm x 20 cm, 250 μm layer, AL SIL G) and #1 filter paper were purchased from Whatman Ltd. (Kent, England).

Instrumentation

The HPLC system included a Varian 9010 solvent delivery system (Varian, Walnut Creek, CA), a Waters WISP 710B sample injector (Waters, Milford, MA), an Ultrasphere ODS C18 reversed-phase column (25 cm x 4.6 mm i.d., 5 μm particle size) (Beckman, Fullerton, CA) equipped with a 7.5 x 4.6 mm guard column (Alltech

Associates, Deerfield, IL), and a Varian 9050 variable wavelength UV-VIS detector (Varian, Walnut Creek, CA). The integration of peaks was completed with Varian Star Chromatography Workstation (Varian, Walnut Creek, CA) software installed on a computer connected to the detector. A Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY) was used for the urinary thiobarbituric acid-reactive substances measurements.

Study population and diets

Complete inclusion and exclusion criteria have been provided by Young et al.[144]. Healthy postmenopausal women with a body mass index between 19 and 32 kg/m^2 were eligible to participate in the original study. Each woman was assigned to complete three study diets in a random order: a high-fat diet (45% energy from fat), a low-fat diet (20% energy from fat), and a low-fat, high n-3 diet (low-fat diet + 3% energy from n-3 fatty acids). The subjects consumed the assigned diet every day for 8 weeks and consumed each of the other study diets for the same period of time following two six- to eight-week washout periods. Study diets were prepared in the metabolic kitchen of the General Clinical Research at the University of Minnesota and distributed to each subject in person. Compliance to the study diets was assessed via daily questionnaires. The study was approved by the University of Minnesota Committee for the Use of Human Subject in Research and the US Army Medical Research and Material Command's Human Subjects Research Review Board.

Twenty-four hour urine collection protocol

As part of the original study, each subject completed two consecutive 24-hour urine collections during the final week of each diet[144]. The samples were kept in an opaque, 3.5 L jug with 1 g ascorbic acid at refrigerator temperature throughout the collection. The collections were pooled, and aliquots were stored at -80°C prior to analyses. The present study includes urine samples from the low-fat (– n-3) and low-fat, high n-3 (+ n-3) diets only. Urinary creatinine was measured for each sample by rate reflectance spectrophotometry using an Ortho Clinical Diagnostics Vitros analyzer at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories.

Urinary thiobarbituric acid-reactive substances (TBARS)

Urinary TBARS were conducted in duplicate for each sample following the method by Lee et al.[145]. Three mL of 5% (w/v) trichloroacetic acid and 1 mL of 0.6% (w/v) thiobarbituric acid were reacted with 1 mL serial dilutions of urine samples, malondialdehyde (MDA) standard, or water (as reagent blank). The mixtures were incubated at 80°C for 90 minutes. The absorbance of the clear supernatant was measured at 535 nm against the reagent blank, and results are computed as MDA equivalents.

Preparation of DNPH reagent

The DNPH reagent was prepared daily following the method in Kim et al.[143]. 12.5 mg of DNPH, recrystallized three times, was mixed the 25 mL of 1 N hydrochloric

acid at 50°C for approximately one hour. Following cooling, the reagent was rinsed four times with hexane to remove any impurities.

Preparation of DNP-hydrazone standards

Four mL of pure standard were combined with 800 mg DNPH, 80 mL methanol, and 2 mL 6 N hydrochloric acid and mixed for 10 minutes at 60°C. After overnight cooling, the solution was filtered and recrystallized three times to purify. The final DNP-hydrazone was dried for two to three days prior to use.

Synthesis and isolation of DNP-hydrazones of urinary aldehydes and related compounds

Three mL of urine were reacted with an equal volume of DNPH reagent overnight at room temperature. A reagent blank and acetone-DNPH standard were prepared by reacting an equal amount of DNPH reagent or 1% acetone/water (v/v), respectively, and DNPH reagent. The following day, the reaction mixtures were extracted three times with 10 mL methylene chloride, and the organic phases were separated via centrifugation for 10 minutes at 1360 x *g*. The sample extracts were pooled and evaporated to 500 μL under N_2 gas. Each sample was applied to two thin-layer chromatography (TLC) plates, and the plates were developed with methylene chloride at room temperature for approximately one hour for primary separation.

Nonpolar and polar compounds were isolated by comparison to the R_f values of acetone-DNPH (0.55) and DNPH reagent (0.23). The nonpolar carbonyl compounds (alkanals, alkenals, ketones, dienals) were found between R_f 0.55 and the solvent front,

while the polar compounds (containing hydroxyl groups) were found between the solvent origin and R_f 0.23. Osazones were isolated between the acetone-DNPH and DNPH reference bands and were discarded from the following analyses. The polar and nonpolar carbonyls were cut from the TLC plates and eluted three times with 10 mL 100% methanol. The pooled extracts were evaporated to approximately 5 mL under N_2 gas and centrifuged for 20 minutes at 1360 x *g* to remove any silica. The clear supernatants were evaporated to less than 1 mL and made up to exactly 1 mL with 100% methanol.

Identification of DNP-hydrazones

Prior to injection into the HPLC system, the methanol extracts were filtered through a 0.45 μm filter. Aliquots of the nonpolar and polar DNP-hydrazones were analyzed separately via HPLC with two different solvent systems[36]. 100 μL aliquots of nonpolar carbonyls were injected into the HPLC system, using isocratic elution for 10 minutes with 75% methanol (v/v), followed by a linear gradient of 75% methanol (v/v) for 20 minutes and 100% methanol (v/v) for 10 minutes. The analysis of polar carbonyls was identical, but the initial isocratic gradient was 55% methanol (v/v). The absorbance of polar and nonpolar carbonyls and related compounds was monitored at 378 nm, and the rejection of peaks was set to 2000 area counts. The detection limit of the system was 1 ng hexanal-DNPH per 50 μL injection. Peaks were identified by comparison of retention times to known standards and previous results from this technique. Each sample was injected at least twice, and paired samples were run in the same series, whenever possible.

Quantification of total urinary mercapturic acids

Following the method of Kress and Pentz[146] as detailed by Kubo et al.[147], 3 mL of urine was acidified with HCl to a pH of 1-2, and the organic components were extracted with ethyl acetate. The extracts were evaporated to dryness under N_2 gas and reconstituted to exactly 3 mL with water. The samples were hydrolyzed with the addition of 900 µL 13.3 N NaOH and one hour incubation at 96°C. Eight hundred µL 0.1 M phosphate buffer (pH 8), 200 µL freshly prepared ascorbic acid solution (2 mg/mL), and 100 µL 1-chloro-2,4-dinitrobenzene (CDNB) solution (0.1 M in DMSO) were added to 1 mL of hydrolyzed urine, and the samples were incubated for 30 minutes at 56°C. Once cooled to room temperature, 150 µL HCl were added to each sample, followed by extraction with diethyl ether.

To quantify total urinary mercapturic acids as *N*-acetyl cysteine, 100 µL of the aqueous phase were injected in to the HPLC system and eluted with an isocratic gradient of 23 mM ammonium formate buffer/acetonitrile (80/20, w/v) at a flow rate of 0.8 mL/min. *N*-acetyl cysteine-CDNB was detected at 340 nm, and the limit of detection was 74 ng *N*-acetyl cysteine-CDNB per 100 µL injection.

Liquid chromatography-mass spectrometry (LC/MS) analysis of 4-hydroxy-2-trans-*decenal (HDE)-DNPH adduct*

Representative subjects' following the + n-3 diet (n=8) DNP-hydrazones were pooled in duplicate, and two samples were collected between 32 and 35 minutes of the polar HPLC method described above. LC/MS analysis was completed by Chi Chen

following his previously published technique[148]. A 5-μL aliquot of the pooled samples was injected into a Waters Acquity ultra-performance liquid chromatography system (Milford, MA) and separated by a gradient of mobile phase ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a ten minute run. LC eluate was introduced into a Waters SYNAPT QTOF mass spectrometer (Milford, MA) for accurate mass measurement and tandem MS (MS/MS) analysis. Capillary voltage and cone voltage for electrospray ionization was maintained at −3 kV and −35 V for negative mode detection, respectively. Source temperature and desolvation temperature were set at 120 and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h) and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50–1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M - H]^-$ = 554.2615 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format. The presence of HDE-DNPH in the samples was confirmed by a comparison with the authentic standard and MS/MS fragmentation.

Statistical analysis

Data were log-transformed and analyzed in SAS v9.2 (SAS Institute Inc., Cary, NC) via paired Student's t-test. The cutoff for significance was set at $p = 0.05$.

Results

Subject characteristics

A summary of baseline characteristics for the subjects is shown in Table 7. The average age of the subjects was 56.21 ± 5.72 years, and the average BMI was 27.70 ± 3.38 kg/m2. Baseline diet information was collected via food frequency questionnaire for 14 of the 15 subjects, and daily diet data are included in Table 7. Compliance during the study was monitored via daily questionnaires and was determined to be less than 1% deviation in energy and less than 0.5% deviation in n-3 fatty acids[144].

Table 7. Baseline demographic and dietary characteristics of healthy women participating in n-3 crossover feeding study.

Baseline characteristic (n=15)	**Mean ± SD**
Race/ethnicity, n (%)	
African-American	1 (6.67%)
Hispanic-White	1 (6.67%)
White	13 (86.67%)
Age, y	56.21 ± 5.72
Body mass index, kg/m^2	27.70 ± 3.38
Systolic blood pressure, mmHg	119.07 ± 15.59
Diastolic blood pressure, mmHg	72.07 ± 7.49
Follicle stimulating hormone, IU/L	72.77 ± 21.89
Baseline diet (n=14)	**Mean ± SD**
Total energy, kcal	1785.11 ± 668.08
Protein, g (% energy)	72.23 ± 30.24 (15.94 ± 1.83%)
Carbohydrate, g (% energy)	229.38 ± 86.52 (51.67 ± 5.56%)
Total fat, g (% energy)	66.06 ± 26.18 (33.41 ± 4.16%)
Saturated fat, g (% energy)	22.10 ± 9.34 (11.18 ± 2.24%)
Monounsaturated fat, g (% energy)	24.65 ± 10.16 (12.43 ± 1.85%)
Polyunsaturated fat, g (% energy)	14.35 ± 10.16 (7.28 ± 0.69%)
18:2n-6, g	12.87 ± 4.69
18:3n-3, g	1.19 ± 0.46
20:4n-6, g	0.086 ± 0.048
20:5n-3, g	0.021 ± 0.037
22:6n-3, g	0.055 ± 0.037
Dietary fiber, g	18.77 ± 7.22
Cholesterol, mg	174.61 ± 85.90

Urinary TBARS

There were no significant differences in urinary TBARS between the diets (Table 8). Urinary MDA equivalents were marginally increased with the n-3 supplemented diet, but this increase was not statistically significant (p = 0.55). The inability to reach statistical significance may be due to the relatively small sample size (n=15) and limited sensitivity and specificity of the TBARS method.

Table 8. Urinary thiobarbituric acid reactive substances (TBARS) following each 8-week diet.

Diet	**+ n-3**	**– n-3**	***p* for difference***
MDA Equivalents (µg/mg creatinine) (geometric mean ± SE)	$9.07 \times 10^{-12} \pm 1.12 \times 10^{-12}$	$8.49 \times 10^{-12} \pm 1.07 \times 10^{-12}$	0.55

Abbreviation:
MDA = malondialdehyde
**p*-value determined via paired t-test for lognormal distribution

Total polar and nonpolar DNP-hydrazones

The total area under the peaks measured from the polar and nonpolar HPLC systems are shown in Figure 11. Following the n-3-supplemented diet, urinary total polar DNP-hydrazones were significantly increased more than two-fold compared to the diet without n-3 supplementation ($p < 0.05$). In contrast, there were no significant differences in urinary nonpolar DNP-hydrazones between the two diets ($p = 0.78$). Although the total concentrations of nonpolar DNP-hydrazones was slightly higher, the significant and substantial increase in polar DNP-hydrazones confirms the hypothesis that moderate n-3 consumption increases total *in vivo* lipid peroxidation, as these polar aldehydes and ketones represent the reactive products of n-3 peroxidation *in vivo*.

.

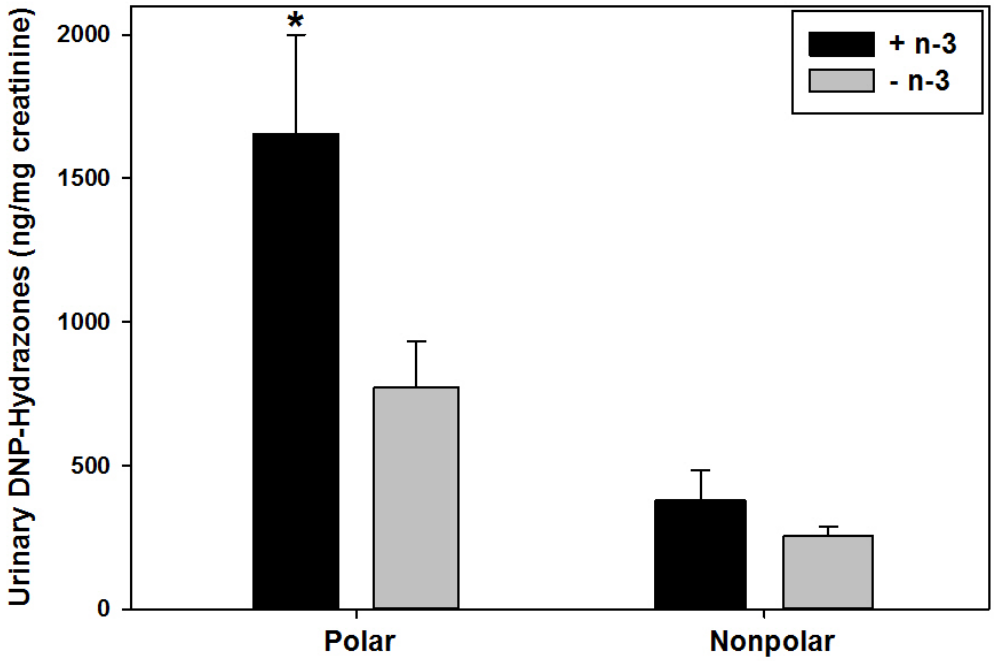


Figure 11. Total urinary polar and nonpolar lipophilic aldehydes following each 8-week diet.
*p <0.05 for paired t-test for lognormal distribution

Total mercapturic acids

Despite the significant increase in polar DNP-hydrazones, there was no significant effect of n-3 supplementation on total urinary *N*-acetyl cysteine (Figure 12).

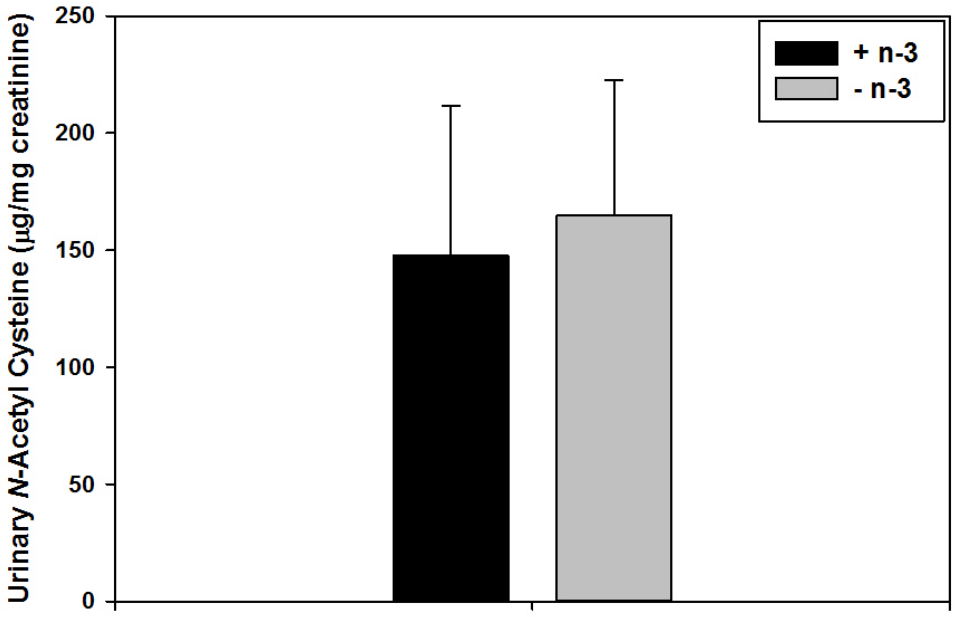


Figure 12. Total urinary *N*-acetyl cysteine (NAC) following each 8-week diet.

Individual polar and nonpolar DNP-hydrazones

Representative chromatograms are shown in Figure 13 and Figure 14. The mean values for individual polar and nonpolar DNP-hydrazones are shown in Figure 15 and Figure 16, respectively. The urinary concentrations of HHE, HDE, and unidentified compounds E and G were significantly higher following the + n-3 diet compared to the – n-3 diet (p <0.05). Additionally, 4-hydroxyoct-2-*trans*-enal (HOE) concentrations were borderline increased with the + n-3 diet (p <0.10). Although all nonpolar compounds quantified in the urine were slightly increased in the + n-3 versus – n-3 diet, none of the differences in concentrations reached statistical significance.

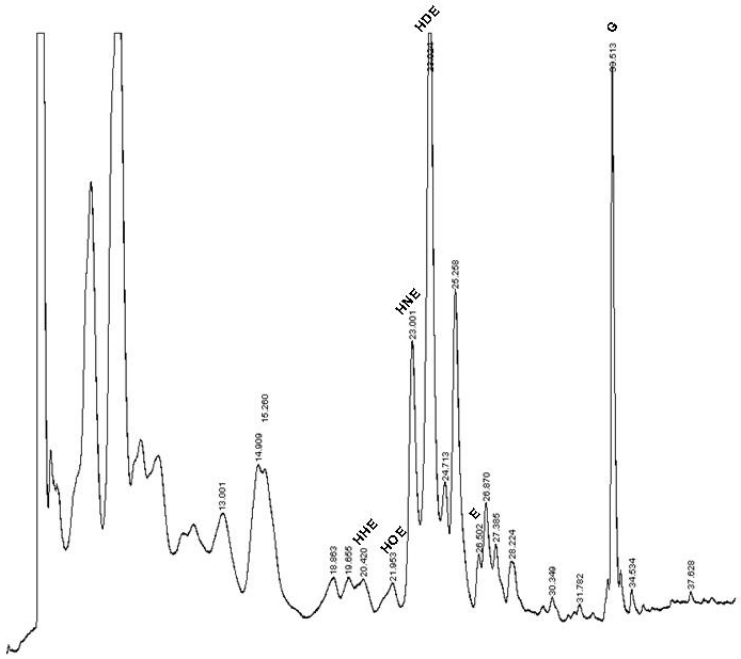


Figure 13. Representative HPLC chromatogram showing polar DNP-hydrazones isolated from human urine.

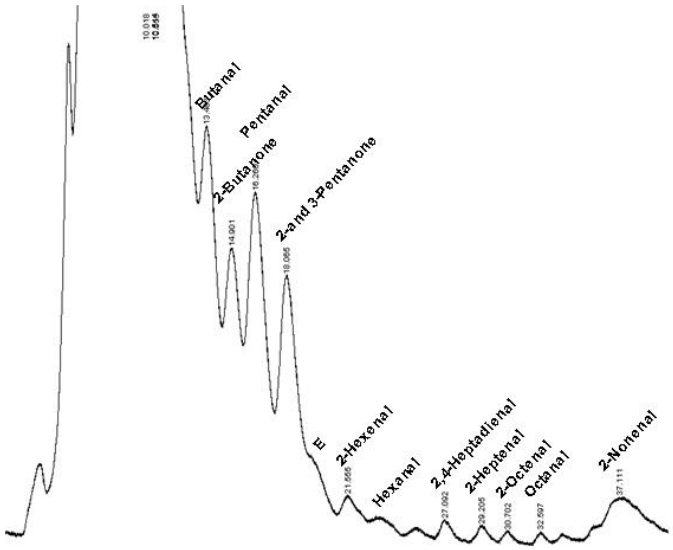


Figure 14. Representative HPLC chromatogram showing nonpolar DNP-hydrazones isolated from human urine.

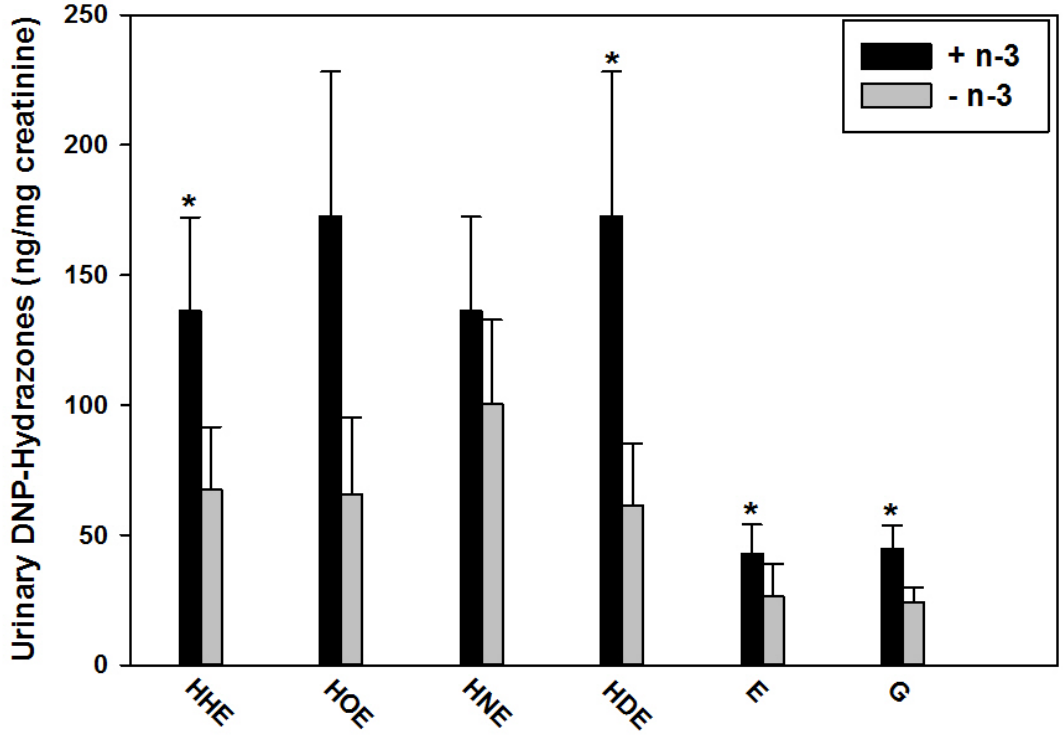


Figure 15. Individual polar urinary DNP-hydrazones following each 8-week diet.
*p <0.05 for paired t-test for lognormal distribution

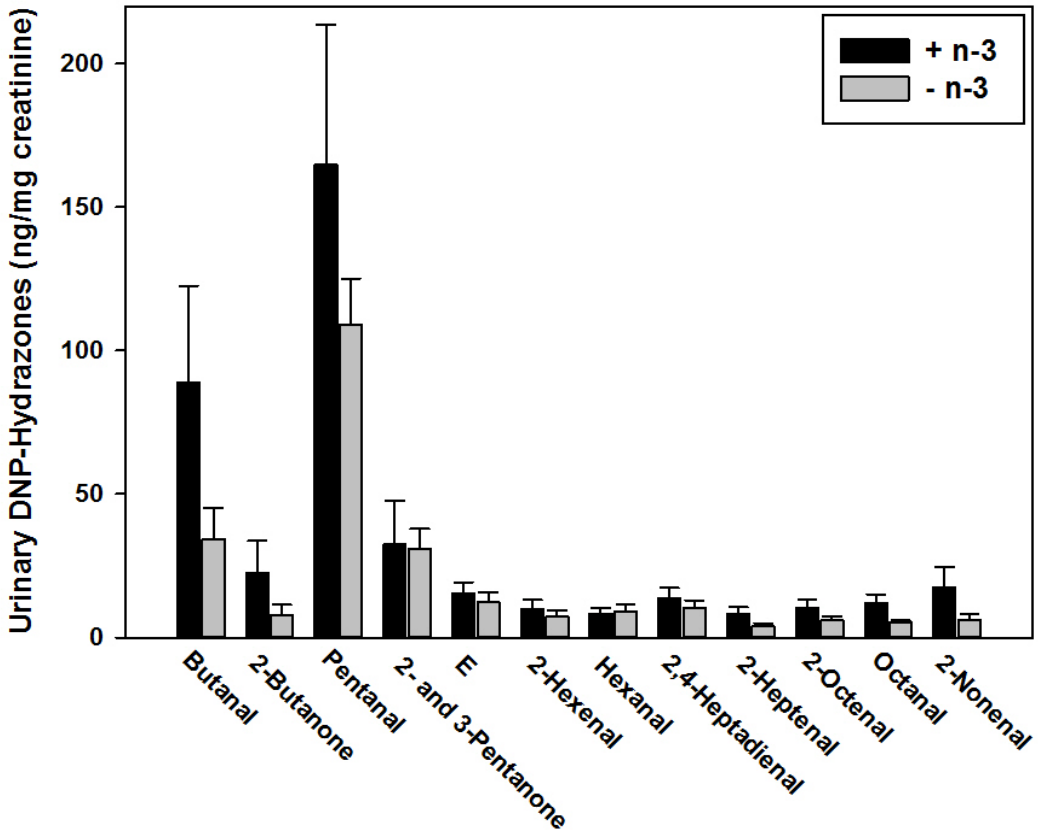


Figure 16. Individual nonpolar urinary DNP-hydrazones following each 8-week diet.

Identification of HDE

Figure 17 shows LC/MS traces confirming the presence of HDE in the urine samples of women consuming n-3. As stated above, the concentrations of HDE were significantly increased following the + n-3 diet ($p < 0.05$). This result marks the first published demonstration of HDE produced from *in vivo* lipid peroxidation of dietary PUFA in human beings.

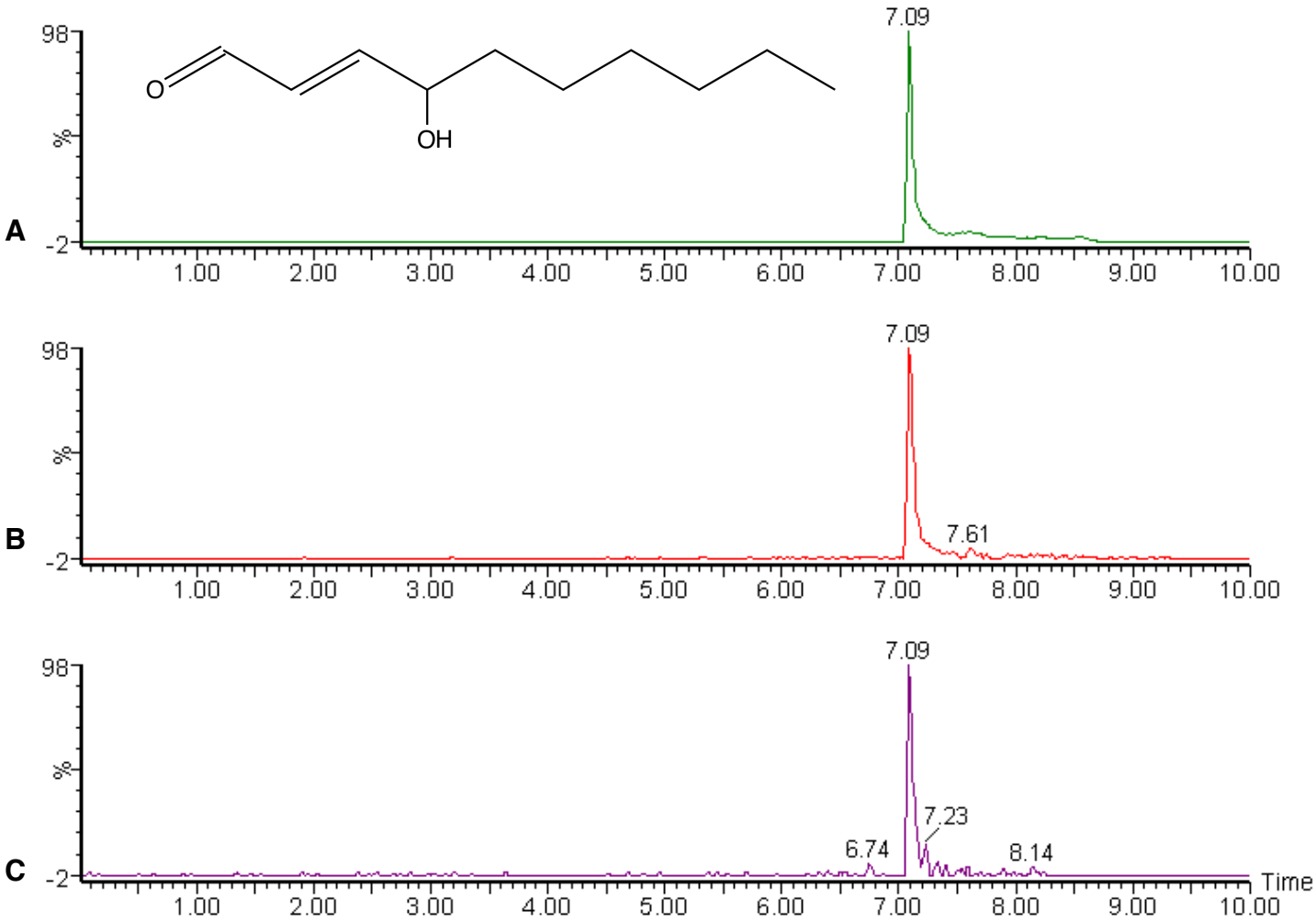


Figure 17. LC/MS trace of 4-hydroxy-2-*trans*-decenal-2,4-dinitrophenylhydrazone (HDE-DNPH) standard (A, with HDE structure), and pooled + n-3 urine samples (B and C) (chromatograms generously provided by Chi Chen).

Discussion

This is the first study to measure a significant increase in total urinary polar DNP-hydrazones and HHE in individuals following a low-fat diet supplemented with 3% of energy from n-3. These results confirm the hypothesis that a moderate increase in dietary n-3 increases *in vivo* lipid peroxidation. Because n-3 PUFA are highly unsaturated, they are susceptible to lipid peroxidation, as shown in previous work by our lab[36]. Although the findings were statistically significant, more research is needed to ascertain the clinical significance of this increase.

Previous human studies have generally shown a significant increase in plasma, low-density lipoprotein (LDL), or urinary TBARS with fish oil or n-3 supplementation[24, 45-49, 52, 53], but our results may not have achieved statistical significance due to the relatively small concentration of n-3 provided to the study subjects as well as the small number of subjects (n=15). In addition, the TBARS method has relatively poor sensitivity and specificity compared to HPLC methods and can result in false positives due to the reaction of thiobarbituric acid with urinary components such as amino acids, sugars, and albumin in addition to MDA[149]. Meydani et al.[46] detected a significant increase in plasma TBARS of women supplemented with more than 2 g of EPA and DHA daily after two months, but this difference was no longer significant at the third month. In a study of men only, Nair et al.[47] documented a significant increase in plasma TBARS following a 10-week treatment with 15 g fish oil per day versus 15 g of placebo oil. Wander et al.[45] supplemented post-menopausal women with the same level of fish oil with or without various concentrations of vitamin E and measured a

significant increase in urinary TBARS after five weeks. In the latter two studies, increased vitamin E intake attenuated the increase in TBARS from high n-3 intake. However, with supplementation of 2.43 g EPA for two weeks, Saito et al.[51] did not detect a significant increase in plasma TBARS among hyperlipidemic women. In the present study, subjects consumed approximately 6.82 g n-3 daily, of which only 0.46 and 0.99 g were EPA and DHA, respectively. This relatively low amount of long-chain n-3 likely explains the contrast of our TBARS results to the studies providing much higher concentrations of n-3 (up to 15 g/d fish oil).

This study is the first to quantify total urinary lipophilic polar and nonpolar aldehydes and ketones produced from the *in vivo* lipid peroxidation of n-3 and among the first to report the concentrations of specific polar lipophilic aldehydes. We documented significant increases in HHE, HDE, and two unidentified compounds in women consuming 3% of energy from n-3 for 8 weeks. This increase in HHE was expected due to its formation from lipid peroxidation of n-3 and confirmed the results of Calzada et al.[54] showing a significant increase in plasma HHE following DHA supplementation at 800 or 1600 mg per day in men. Similar to Turley et al.[50], we did not detect a significant increase of HNE with n-3 supplementation. This is not surprising given that thermal oxidation of ALA did not produce quantifiable levels of HNE, in contrast to linoleic acid (n-6)[36]. As discussed below, this is the first study to document an increase in HDE excretion from *in vivo* lipid peroxidation of dietary n-3, and additional human studies are required to confirm this result. In addition, further research is needed to identify unknown compounds E, and G, but it is plausible that they are long chain (at

least ten carbons) polar lipophilic aldehydes produced from lipid peroxidation of n-3. We failed to quantify any significant changes in total or individual nonpolar lipophilic aldehydes following the n-3 diet treatment. Although not significant, there were increased concentrations of these compounds following the n-3 diet, suggesting an overall increase in their production, likely due to increased substrate availability.

We did not measure significant differences in urinary *N*-acetyl cysteine concentrations following the two diets, indicating the low-fat diet supplemented with n-3 did not change total mercapturic acid excretion. However, this method did not quantify specific mercapturic acid conjugates, which may be influenced by other environmental exposures. Nevertheless, our results are supported by those of Kubo et al.[147], who did not detect a statistically significant difference in total urinary mercapturic acid excretion between rats fed a standard diet or a diet supplemented with 8.4% of energy from DHA. These results suggest that either intracellular glutathione has been saturated or higher concentrations of n-3 are required to enhance this method of detoxification. The work of Kuiper et al.[89] suggests that substantially enhanced lipid peroxidation is required to increase urinary mercapturic acid excretion, as these researchers documented significant increases in mercapturic acid conjugates of lipid peroxidation metabolites in rats treated with CCl_4, a potent inducer of oxidative stress.

Through highly sensitive LC/MS analysis, we were able to confirm significantly increased concentrations of HDE following the + n-3 diet. This is the first study to document urinary excretion of HDE from dietary fat intake as well as its significantly increased concentration following a diet containing 3% of calories from n-3. These

results suggest that HDE is produced from lipid peroxidation of n-3. Although research examining HDE is limited, it is plausible that this compound shares the abilities of HHE and HNE to bind proteins and DNA, potentially resulting in cellular mutations and death. More research is needed to confirm this finding in humans as well as establish its clinical relevance.

In conclusion, we demonstrated that a low-fat diet supplemented with 3% of energy from n-3 significantly increased total polar lipophilic aldehydes from *in vivo* lipid peroxidation. At present, it is not known whether this increase is clinically significant. The absence of an increase in mercapturic acid excretion may indicate that the increase of *in vivo* lipid peroxidation falls well below toxic levels, but slight increases in the production of reactive aldehydes such as HHE may cause cellular damage leading to neurodegenerative diseases, atherosclerosis, or cancer[35, 39, 40, 58-60, 75, 150-153]. Future research utilizing highly sensitive techniques to characterize urinary lipid peroxidation metabolites from diets with a range of n-3 concentrations will help establish at what doses n-3 are no longer beneficial and may become detrimental to human health.

Part II: Validation of an assay for the measurement of 3,3'-diindolylmethane as a marker for indole-3-carbinol exposure in free-living subjects

Introduction

There is ample evidence that consuming cruciferous vegetables, such as broccoli, cauliflower, or Brussels sprouts, reduces the risk of several human cancers. In addition to macronutrients, vitamins, and minerals present in these vegetables, crucifers are rich sources of glucosinolates, which generate bioactive compounds during metabolism. Indole-3-carbinol is a metabolite of the glucosinolate glucobrassicin and has demonstrated substantial chemoprevention in cell culture and animal model studies of cancers. However, the lack of a reliable biomarker of indole-3-carbinol exposure in humans has limited epidemiological research in this area. The development and validation of a urinary assay for 3,3'-diindolylmethane, the principle metabolite of indole-3-carbinol in humans, would prove valuable for human research of indole-3-carbinol exposure and cancer risk.

Chapter III: Literature Review

Cruciferous vegetables and cancer risk

Cruciferous vegetables largely consist of the *Brassica* genus of vegetables, such as cabbage, Brussels sprouts, broccoli, cauliflower, turnips, and bok choy, but also include non-*Brassica* vegetables such as radishes, watercress, horseradish, and arugula[154]. These vegetables are characterized by bitter, pungent flavors and aromas due to their high concentrations of sulfur-containing compounds called glucosinolates[155]. Upon damage to the plant cells by processing or mastication, the enzyme myrosinase is released, resulting in the hydrolysis of glucosinolates (Figure 18)[156]. The hydrolysis products, which include isothiocyanates and indoles, are the putative bioactive constituents of cruciferous vegetables that exert health effects *in vivo*[157-159].

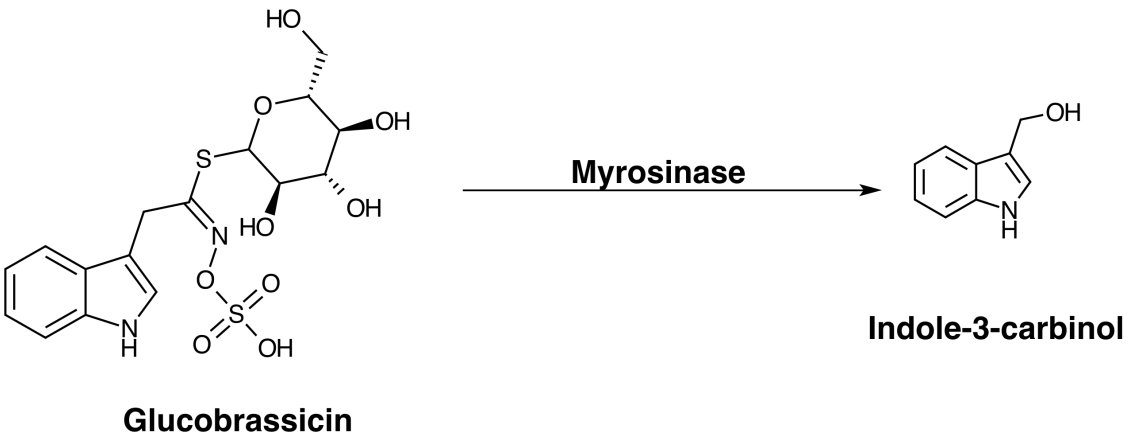


Figure 18. Conversion of parent glucosinolate, glucobrassicin, to indole-3-carbinol (I3C), catalyzed by myrosinase.

There has been extensive epidemiological research into cruciferous vegetables and decreased risk of several human cancers, particularly lung cancer (reviewed in [154, 156, 158, 160, 161]). Although they are also a source of dietary fiber and several vitamins and minerals including the antioxidant selenium, the chemopreventive effects of cruciferous vegetables are credited primarily to the modulation of phase I and II xenobiotic metabolism enzymes by isothiocyanates and indoles[154]. Phase I metabolism is generally considered an activating step for pro-carcinogens, while phase II metabolism is responsible for the conjugation of carcinogens and other xenobiotic compounds to increase water solubility and excretion via urine or feces (Figure 19)[154, 162]. Therefore, a dietary bioactive that increases the activity of these enzymes would decrease the exposure of an organism to a carcinogen, thereby reducing cancer risk. Additional chemopreventive mechanisms specific to indoles, including the up-regulation of apoptosis, will be discussed in the next section.

Much nutrition and cancer research has focused on total and specific isothiocyanates generated from hydrolysis of glucosinolates because of the existence of sensitive and specific urinary biomarkers[163]. Specifically, phenethyl isothiocyanate (PEITC, generated from watercress glucosinolates) and sulforaphane (SFN, generated from broccoli glucosinolates, among others) have been extensively studied with respect to human cancers[164]. Their effects on chemoprevention have been largely attributed to the up-regulation of phase II metabolizing enzymes, but there is some evidence that SFN may decrease the activity of phase I metabolizing enzymes, reducing the activation of pro-carcinogens[164]. Intriguingly, SFN may also enhance formation of the lipid

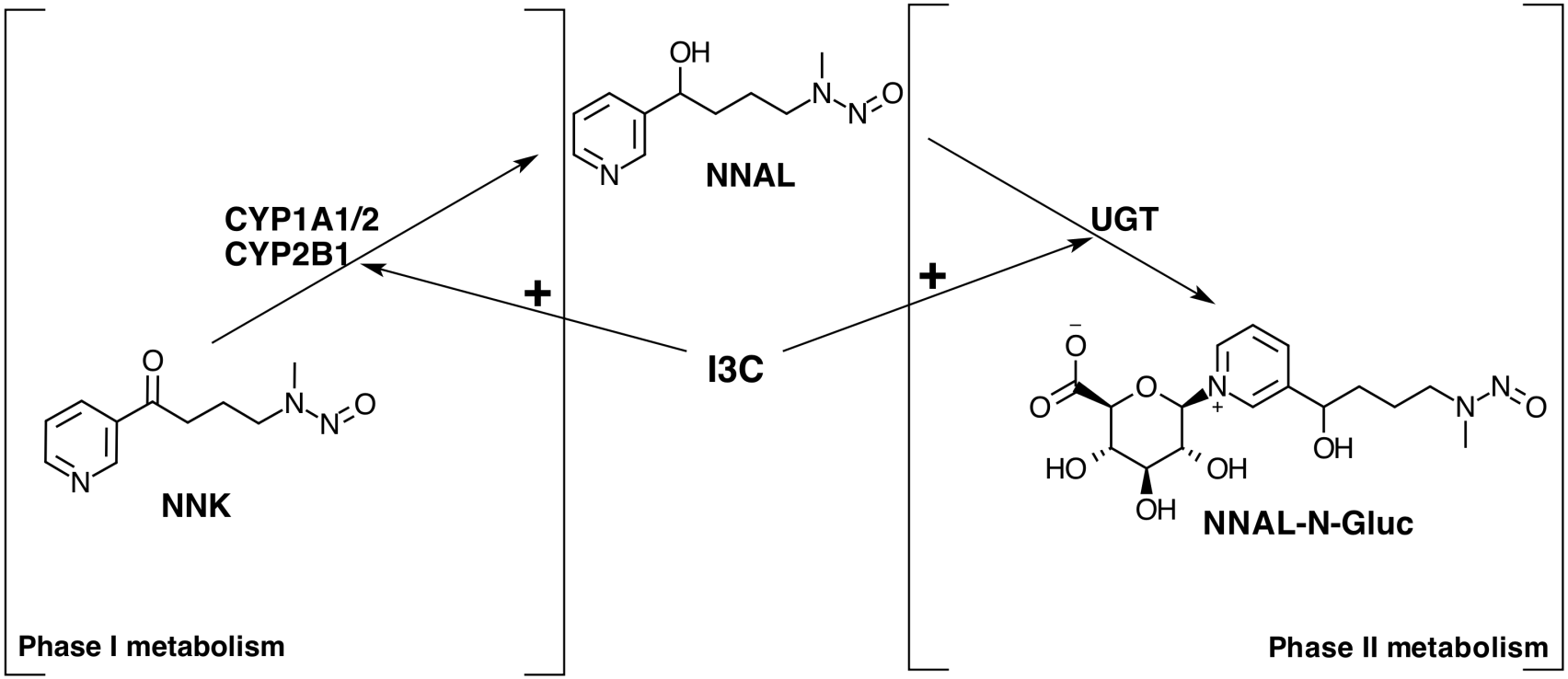


Figure 19. Example of Phase I and II metabolism of the tobacco-specific carcinogen 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) into 4-(methylnitrosamino)-1-(3-pyridyl-1-butanol) (NNAL), catalyzed by cytochrome P450 (CYP) isoforms 1A1, 1A2, or 2B1, and NNAL-N-glucuronide (NNAL-N-Gluc), catalyzed by UDP-glucuronosyltransferase (UGT). Both enzymes have been shown to be up-regulated by indole-3-carbinol (I3C) (adapted from [157, 161, 165, 166]).

peroxidation-derived α,β-unsaturated aldehyde HNE to increase cancer cell apoptosis[167]. However, the chemopreventive effects of indoles are more appealing from a therapeutic and public health standpoint because they have been shown to modulate both phase I and phase II metabolism with greater potency and less toxicity than isothiocyanates[154, 158, 162, 168, 169].

Metabolism of indole-3-carbinol

As previously mentioned, processing of cruciferous vegetables through chewing or chopping releases myrosinase, which cleaves the glucose moiety from the glucosinolate[158]. Indole-3-carbinol (I3C) is formed following the degradation of glucobrassicin by myrosinase. Following ingestion, the low pH of the gastric cavity promotes the dehydration and oligomerization of I3C into several metabolites (Figure 20)[154, 170, 171]. Of these metabolites, 3,3'-diindolylmethane (DIM) is the most prevalent and stable in body tissues; therefore, it is a promising candidate for a biomarker of I3C exposure[170, 171]. Relatively little is known about the metabolic fate and excretion of DIM. It is plausible that the compound is excreted in the urine as an N-glucuronide following conjugation via UDP-glucuronosyltransferases (UGTs)[172]. Additionally, DIM may be excreted as a sulfonated conjugate, produced by the phase II sulfotransferase enzymes (SULTs)[173, 174]. The pharmacokinetics of I3C metabolism and DIM formation in animals and humans will be reviewed below.

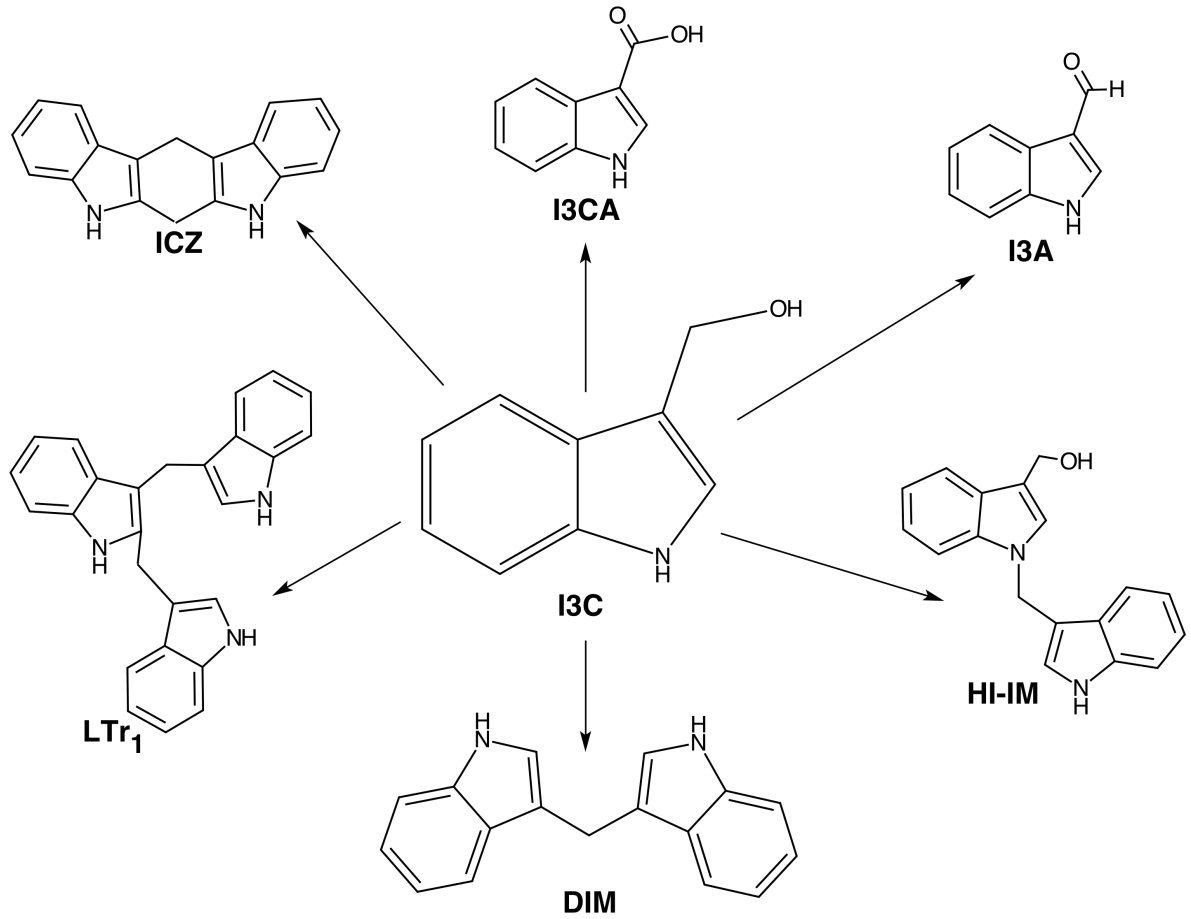


Figure 20. Acid condensation of indole-3-carbinol (I3C) into 3,3'-diindolylmethane (DIM) as well as [2-(indol-3-yl-methyl)-indol-3-yl]indol-3-ylmethane (linear trimer, LTr_1), indolo[3,2b]carbazole (ICZ), indole-3-carboxylic acid (I3CA), indole-3-carboxaldehyde (I3A), and 1-(3-hydroxymethyl)-indolyl-3-indolylmethane (HI-IM) (adapted from [171]).

Indole-3-carbinol, 3,3'-diindolylmethane, xenobiotic metabolism, and cancer risk

In vitro *studies*

A majority of primary research into I3C, DIM, and human cancers has occurred in cell culture systems, and a selection of relevant studies are summarized in Table 9. To explore the apoptotic mechanism in the single cell fungal organism *Candida albicans*, Hwang et al.[175] supplemented cells with 1% I3C with or without inhibitors of oxidative stress and measured the generation of ROS and hydroxyl radicals and markers of apoptosis and necrosis. The authors concluded that I3C indeed acts through an oxidative stress mechanism, chiefly an increase in the production of the hydroxyl radical, to stimulate apoptosis and possibly necrosis of *C. albicans*. Such effects of I3C may be similar to the effects of lipid hydroperoxides to prevent cancer progression (see Chapter 1), but more research is needed to demonstrate this.

Many studies examining I3C and human cancers have focused on the transcriptional effects of the dietary bioactive. Marconett et al.[176] treated estrogen receptor-positive human breast cancer cells with varying concentrations of I3C and concluded that I3C decreased cell proliferation by down-regulating the transcription of multiple growth-inducing genes. I3C treatment was shown to interfere with the interaction between estrogen receptor-α and the transcription factor Sp1 via phosphorylation of a key amino acid on the molecule, providing a possible explanation for the transcriptional effects of I3C.

In a study of human prostate cancer cells, Wang et al.[177] showed that treatment of cells with either I3C or DIM significantly decreased proliferation of androgen-

Table 9. Summary of *in vitro* studies examining indole-3-carbinol, 3,3′-diindolylmethane, and cancer.

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
Hwang et al.[175]	*Candida albicans* Untreated cells as controls	1% I3C (with or without thiourea or trehalose as inhibitors of oxidative stress), amphotericin B, or hydrogen peroxide	Minimum inhibitory concentration ROS and hydroxyl radical concentration via fluorescent dye Apoptosis and necrosis via Annexin V staining and TUNEL staining Cytochrome C via spectrophotometry and SDS-PAGE Mitochondrial membrane potential via flow cytometry Metacaspase activation via kit	I3C was as efficient as amphotericin B or hydrogen peroxide in anti-candidal activity. I3C did not increase cellular ROS as much as amphotericin B or hydrogen peroxide but did increase hydroxyl radical production. I3C stimulates apoptosis or discrete necrosis and DNA condensation and fragmentation via an oxidative stress mechanism. I3C increases cytochrome C release from mitochondria, which was not inhibited with protection against oxidative stress. I3C also increases metacaspase activation, stimulating apoptosis.	
Jin[178]	MCF-7 and MDA-MB-468 human breast cancer cells with transfected Cdc25A, a phosphatase involved in cell cycle progression Untreated cells as controls BALB/C female nude mice (n=40) with or without human breast cancer cell xenograft	30, 45, or 60 μM DIM for 24-96 h For mouse study, 5 mg/kg/d DIM or sesame oil as control 5 d each week for 7 weeks	Cell proliferation via cell counting Colony formation via staining miRNA target prediction via computer programs Growth inhibition via anti-miR-21 or miRNA negative control treatment and cell counting Protein expression via Western blot Cell cycle progression via flow cytometry	DIM inhibited the growth and proliferation of breast cancer cells in a time- and dose-dependent manner. DIM treatment significantly decreased tumor formation by 60% in xenograft study (p <0.05). DIM treatment increased the proportion of MCF-7 cells in G_1 and G_2/M phases of the cell cycle; MDA-MB-468 cells treated with DIM were arrested in the G_2/M phase. DIM decreased the expression (but not transcription) or CDK4 and CDK2 in MCF-7 cells and increased $p21^{Waf1/Cip1}$ in both cell lines. DIM treatment was also shown to down-regulate Cdc25A, Cyclin B1, and CDK1 expression in both cell lines. The Ser^{124} residue on Cdc25A is likely responsible for effect of DIM, shown via miRNA target prediction.	MCF-7: estrogen receptor-positive, wild-type *p53* MDA-MB-468: estrogen receptor-negative, *p53* mutant

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
				Cells treated with anti-miR-21 and DIM had increased proliferation over cells treated with DIM alone, indicating the miR-21 is involved in the effect of DIM on breast cancer cell proliferation.	
Marconett et al.[176]	MCF-7 human breast cancer cells, with or without transfection of *hTERT*, which is involved in telomerase activity Untreated cells as controls	50, 100, 150, 200, or 250 μM I3C for 48 h 200 μM I3C for 72 h	Gene expression via RT-PCR Cell cycle progression via flow cytometry Telomerase activity via kit Cell senescence via fluorescent microscopy Binding of hTERT to ERα and Sp1 via chromatin immunoprecipitation	I3C decreases *hTERT*, *ERα*, *CDK6*, and *progesterone receptor* in dose-dependent manner. Down-regulation of *hTERT* correlated with the proportion of cells in the G_1 phase of the cell cycle. Cells transfected with *hTERT* were resistant to this effect of I3C. I3C treatment decreased telomerase activity; again, this effect was ablated in the transfected cells. Treatment with I3C significantly increased cellular senescence ($p = 0.0225$), and this result was absent in transfected cells. Decrease in *ERα* and *CDK6* were detected prior to decrease in *hTERT*, suggesting that hTERT expression may decrease as a response to the down-regulation of ERα and CDK6 by I3C. I3C treatment disrupted the interactions between ERα and Sp1 on their response elements located on the *hTERT* promoter. I3C was shown to increase phosphorylation of Sp1 at Thr^{579}.	
Saati and Archer[179]	MCF-7, MDA-MB-231, and SKBr-3 human breast cancer cells MCF-10A nontumorigenic	50 μM DIM	Cell proliferation via MTT assay Fatty acid synthase and Sp1 protein expression via Western blot	Treatment with DIM significantly decreased the proliferation of all cancer cells at all time points ($p < 0.01$). DIM had no effect on proliferation of the nonmalignant cells. DIM treatment for at least 24 h significantly decreased the levels of fatty acid synthase and Sp1 in MCF-7 cells ($p < 0.01$). There	Cancers frequently overexpress fatty acid synthase. Plasma DIM of 24 μM and

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
	human breast epithelial cells Also untreated breast cancer cells as controls			were significant reductions in expression of these proteins in MDA-MB-231 and SKBr-3 cells after 48 h of DIM treatment (p <0.01). There were no changes in the expression of these proteins in nonmalignant breast cells treated with DIM.	tissue DIM of 32-200 μM reported following oral supplementation of 250 mg/kg DIM by mice.
Lerner et al.[180]	HCT-116 and Colo-320 human colon cancer cells Untreated cells as controls	10-100 μM DIM for 24, 48, or 72 h	Cell viability and cytotoxicity Toxicity via lactate dehydrogenase leakage DNA damage via DAPI staining N-myc downstream regulated 1 gene (NDRG1) expression via PCR Cell differentiation via alkaline phosphatase activity	DIM treatment up to 80 μM significantly reduced viability of both cell lines in a dose-dependent fashion (p <0.001). There were no toxic effects of DIM treatment as indicated by leaking of lactate dehydrogenase. Cells treated with 60 μM exhibited signs of apoptosis via DAPI staining after 48 h. Treatment with 60 μM DIM significantly increased *NDRG1* expression in Colo-320 (p <0.001 for all time points) but not HGT-116 cells. DIM treatment had no significant effects on differentiation of either cell lines.	HCT-116 have wild-type *p53*; Colo-320 have mutant *p53*.
Wang et al.[177]	LNCaP and PC-3 human prostate cancer cells Untreated cells as controls	0-25 μM DIM for 0-72 h 0-100 μM I3C for 0-72 h 1 nM dihydrotestosterone or 17β-estradiol for gene expression experiments	Cell growth via sulforhodamine B assay Cell cycle distribution via flow cytometry Gene expression via RT-PCR Protein expression via Western blot Androgen receptor binding affinity via kit Aryl hydrocarbon receptor binding via siRNA knockdown	Both DIM and I3C significantly inhibit the proliferation of LNCaP cells (p <0.05) in a dose-dependent manner. DIM is more potent than I3C (EC50 10 μM and 100 μM, respectively). Only I3C significantly decreased PC-3 proliferation at 100 μM (p <0.05). Both DIM and I3C stimulated G_0/G_1 arrest in a dose-dependent fashion; again DIM was more potent than I3C. At 25 μM, DIM significantly increased *CDKN1A* and *CDKN1B* (p <0.05). I3C exerted the same effects at 100 μM. Only I3C significantly increased the CDKN1A	LNCaP are androgen-dependent whereas as PC-3 are androgen non-responsive cells. This study also compared the effects of DIM and I3C on gene expression to genistein,

Reference	System/Controls	Intervention	Outcome Measurement(s)	Results	Notes
				protein levels (p <0.05), while both DIM and I3C significantly increased CDKN1B (p <0.05) in LNCaP cells. All concentrations of DIM tested significantly decreased the transcription of androgen-dependent proteins (PSA, NKX3.1, and IGF-1R) and expression of PSA (p <0.05) in LNCaP cells. I3C showed similar effects at higher concentrations. Both DIM and I3C significantly increased transcription of *AHR*, *CYP1A1*, and *NQO1* (DIM only) (p <0.05). DIM significantly increased CYP1A1 and NQO1 expression in both cell lines (p <0.05). These effects were blocked with AHR siRNA. In PC-3 cells, DIM and I3C significantly increased *CDKN1A* transcription (p <0.05). *CYP1A1* and *NQO1* were significantly increased in PC-3 cells treated with DIM or I3C (p <0.05). DIM was the only phytochemical tested that bound to the androgen receptor. Both DIM and I3C inhibited the dihydrotestosterone or 17β-estradiol induction of PSA (p <0.05).	SFN, and resveratrol. The effects were similar, but SFN inhibited *CYP1A1* expression.

dependent cells in a dose-dependent manner (p <0.05) and that DIM was more potent than I3C, with a 50% median effective concentration of 10 μM compared to 100 μM, respectively. Further experiments demonstrated that DIM was also more effective than I3C at up-regulating gene expression involved in stimulating cell cycle arrest (*CDKN1A, CDKN1B*) and phase I metabolism (*CYP1A1, AhR, NQO1*); the blockage of these effects through the addition of small interfering RNA for AhR suggested that they are due to the binding of DIM to this transcription factor and subsequent up-regulation of gene expression. This study also compared the effects of I3C and DIM to several other phytochemicals including SFN, which were similar with the exception of SFN inhibiting *CYP1A1* expression.

Although numerous cell culture studies have utilized pure I3C, it is important to note that I3C can spontaneously form DIM at rates in excess of 50% during such experiments even in the absence of low pH[181]. Therefore, it is plausible that DIM accounts for the majority of biological effects of I3C documented in the aforementioned studies. Focusing on treatment with DIM alone, Jin[178] treated human breast cancer cells and a human breast cancer mouse model and detected a dose- and time-dependent suppression of breast cancer growth and proliferation. Both cell lines used showed enhanced cell cycle arrest through decreased expression of cell cycle progression molecules (Cdc25A, Cyclin B1, and CDK1) and increased expression of a cyclin inhibitor, $p21^{Wad1/Cip1}$. Further treatment with a small interfering RNA demonstrated that the effects of DIM on the cell cycle might be due primarily to its up-regulation of a micro-RNA, miR-21. These experiments provide some evidence that DIM is effective at

suppressing breast cancer proliferation irrespective of hormonal dependence and may exert its effects through the activation of a micro-RNA, a burgeoning field of interest in cancer research.

Subsequently, Saati and Archer[179] tested the effects of DIM in several human breast cancer cell lines in addition to a non-tumorigenic human breast cell line and concluded that DIM significantly decreased proliferation of all cancer cell lines (p <0.01) but had no effect on non-tumorigenic cell proliferation. Similar results were seen regarding the decreased expression of FAS and Sp-1 in the cancer but not non-cancer breast cells. The results suggest that the ability of DIM to decrease cell proliferation may be confined to cancer rather than non-tumorigenic tissues, but it is noteworthy that the experiments used a relatively high concentration of DIM (50 μM) for at least 24 hours, and these levels are not achievable through dietary intake of cruciferous vegetables alone.

Lerner et al.[180] tested the effects of various concentrations of DIM on two human colon cancer cell lines with regard to viability, toxicity, DNA damage, differentiation, and gene expression. These researchers concluded that DIM treatment up to 80 μM significantly decreased cell viability in a dose-dependent manner (p <0.001), and 60 μM of DIM resulted in apoptosis after 48 hours. Additionally, the absence of lactate dehydrogenase in the surrounding media suggested that DIM treatment at the median inhibitory concentrations (~54 μM) was not toxic to the cells. Although 60 μM of DIM was shown to increase the transcription of a gene involved in cell differentiation (*NDRG1*) in one of the cell lines, DIM treatment did not show any significant effects on cell differentiation in either cell line.

While each of these cell culture experiments demonstrates the capacity of DIM and I3C to enhance apoptosis, decrease cancer cell proliferation, and cause cell cycle arrest, it is difficult to extrapolate the results to humans or even animal models for several reasons. As mentioned previously, the concentrations of DIM and I3C used are not achievable through dietary consumption of cruciferous vegetables and likely represent pharmacological rather than physiological doses. Consequently, it is impossible to achieve the level of exposure to cells within a whole organism without the use of a high amount of oral supplementation, which is limited by the bioavailability of the supplement as well as possible adverse events, such as gastrointestinal discomfort. Additionally, cell culture systems utilize continuous exposure to the compound of interest, typically over the course of multiple days. Given the continuous metabolic flux within cells and the documented short half-lives of DIM and I3C (see below), the exposure of cells within an organism to these compounds for such a long duration is not plausible. In contrast to lipid hydroperoxides, which are continually present in cells at basal levels (see Chapter 1), dietary bioactives such as DIM and I3C are not endogenously generated and are subject to xenobiotic metabolism and excretion by the very systems they up-regulate. It remains to be seen what effects—if any—a physiological dose of DIM or I3C achieved through the diet has on human cancers, whether in the whole organism or isolated cell culture systems.

Animal model studies of DIM and I3C pharmacokinetics

Because of their potential for therapeutic use, the pharmacokinetic profiles of DIM

and I3C have been evaluated in mice and rats, as summarized in Table 10. Seminal work in this field was conducted by Stresser et al.[170], who administered radio-labeled I3C to rats over a one-week period and measured the presence of I3C and its metabolites in blood and numerous tissues. Once steady-state was achieved, 75% of the I3C dose was excreted in total. Seventy seven percent of the total excreted I3C was detected in feces and 23% was detected in urine. The highest concentrations of I3C were measured in liver tissues, followed by lung and blood. Additionally, the authors identified and quantified several acid condensation products of I3C, including DIM, which was present in high concentrations in the liver relative to other metabolites.

Anderton et al.[182] compared the bioavailability and pharmacokinetics of crystallized and microencapsulated DIM in female mice. Although the microencapsulated formulation of DIM resulted in increased absorption and higher tissue concentrations, both formulations elicited a peak DIM concentration in all tissues between 30 minutes and one hour following administration. This is indicative of the short half-life of DIM. Additionally, the researchers did not detect downstream DIM metabolites in the plasma or tissues, which suggests that DIM likely exerts its biological effects in its native form.

As a corollary study, these researchers performed similar experiments with the administration of the same amount of I3C to female mice[171]. Plasma concentrations of I3C peaked at 15 minutes following gavage, and concentrations fell below the limit of detection within one hour, establishing a very short half-life for I3C. Plasma DIM peaked two hours following gavage, while an additional acid condensation product,

Table 10. Summary of animal model studies examining indole-3-carbinol and 3,3′-diindolylmethane pharmacokinetics.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Stresser et al.[170]	Male Fischer rats fed 0.32% [^{3}H]I3C for 1d then 0.2% [^{3}H]I3C for 6d Pharmacokinetics measured in blood, liver, kidney, lungs, stomachs, small intestines, tongues, urine, and feces	Tissue, blood, urine, and fecal radioactivity via scintillation counting I3C and metabolite identification and quantification via HPLC, NMR, and MS	At steady-state, 77% of I3C excreted via feces and 23% via urine. This represents 75% overall excretion. The highest tissue concentrations of I3C were in the liver, followed by lung and blood. Following gavage, the highest concentrations were in the liver, followed by kidney, lung, blood, and tongue. Six unique I3C metabolites were identified (DIM, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3ylmethane (LTr_1), 1-(3-hydroxymethyl)indolyl-3-indolylmethane (HI-IM), and 3 structurally unidentified compounds).	pH of stomach contents 4.3 ± 0.03.
Anderton et al.[182]	Female CD-1 mice gavaged with 250 mg/kg crystallized or microencapsulated DIM Pharmacokinetics measured in blood, liver, kidney, lung, heart, and brain	Blood and tissue DIM and other I3C condensation products via HPLC	Peak DIM concentration in all tissues 0.5 to 1 h after administration. Highest DIM concentrations observed in the livers, then lungs, kidneys, and hearts. The microencapsulated DIM resulted in higher tissue DIM and increased absorption compared to crystallized DIM. No metabolites of DIM detected in plasma or tissues.	
Anderton et al.[171]	Female CD-1 mice gavaged with 250 mg/kg I3C or 10 ml/kg corn oil as control Pharmacokinetics measured in blood, liver, kidney, lung, heart, and brain	Plasma I3C, DIM, and LT_1 concentrations via HPLC and MS	Plasma I3C peaked at 15 min after dose and fell below the limit of detection with 1 h. Highest I3C concentrations in liver, followed by kidney, plasma, lung, heart, and brain. Peak DIM and LTr_1 concentrations 1/6 and 1/10 those of I3C. DIM peaked at 2 h, while LTr_1 concentrations continued to increase throughout duration of testing (6 h). DIM and LTr_1 concentrations highest in liver, followed by kidney, brain, lung, and heart. HI-IM also identified via HPLC-MS and exhibited similar pharmacokinetics to DIM. DIM, LTr_1, and HI-IM remained in plasma and	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			tissues longer than I3C. ICZ also detected in mouse liver at 6 and 24 h. I3CA detected in plasma via HPLC and had similar pharmacokinetics to I3C. I3A detected in plasma below limit of quantification.	

Abbreviations:
LTr_1 = 2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane
HI-IM = 1-(3-hydroxymethyl)indolyl-3-indolylmethane
ICZ = indolo[3,2b]carbazole
I3CA = indole-3-carboxylic acid
I3A = indole-3-carboxyaldehyde

2-(indol-3-ylmethyl)-indol-3-yl]indol-3ylmethane (LTr_1), continued to increase in concentration throughout the measurement period of 6 hours following gavage. An additional metabolite, 1-(3-hydroxymethyl)indolyl-3-indolylmethane (HI-IM), was present in similar concentrations and exhibited similar pharmacokinetics to DIM. Similar to the previous study, the highest concentrations for I3C and its condensation products were detected in the liver; notably, detectable concentrations of DIM, LTr_1, HI-IM remained in the liver for at least 24 hours following I3C administration. Three additional I3C metabolites were detected: indolo[3,2b]carbazole (ICZ), indole-3-carboxylic acid (I3CA), and indole-3-carboxaldehyde (I3A); however, the authors considered these minor compounds with little physiological relevance, given their very low plasma and tissue concentrations as well as documented diminished ability to affect cell cycle progression compared to I3C and DIM.

While pharmacokinetic studies conducted in rodents may offer valuable information about the metabolism of I3C and DIM within mammals, there are several limitations to such studies. As mentioned regarding *in vitro* studies, the quantities of I3C and DIM given to the test animals far exceed what could be achieved through the human diet. It is likely that such high doses influence the pharmacokinetic profiles and tissue distribution of these dietary bioactives, and the results may not be applicable to controlled feeding studies in humans. Additionally, the authors of these studies acknowledge that a major limitation of such work is the relatively high pH of murine stomach acid compared to that of humans[170]. It is conceivable that such differences in pH would change the amounts and types of acid condensation products formed between murine animals and

humans.

Animal model studies of biological effects of DIM and I3C

Table 11 summarizes studies examining I3C, DIM, and xenobiotic metabolism and/or human cancers in animal model systems. Leibelt et al.[183] administered various quantities of DIM or I3C to male and female Sprague-Dawley rats and monitored changes in liver size and enzymatic activity as well as colonic enzyme activity, bone density, serum vitamin D, and serum testosterone (in male rats only). Male rats fed I3C or the highest dose of DIM had significantly increased liver somatic indices (the percentage of body weight comprised of liver weight) compared to those fed the control diet ($p = 0.0023$ and $p = 0.042$, respectively). In both male and female rats, hepatic and colonic CYP contents were significantly increased by I3C and DIM supplementation; unfortunately, the researchers did not measure CYP activity, which would have indicated if I3C and DIM indeed enhanced the functions of CYP. There were no significant effects of I3C or DIM on bone density or serum testosterone. Additionally, serum vitamin D was significantly increased following one year of I3C supplementation ($p = 0.0371$ in males, $p = 0.0031$ in females). Decreases in several liver enzymes (aspartate aminotransferase, alkaline phosphatase, and creatinine kinase) were detected in male rats, which show the absence of liver toxicity from the dietary compounds. Although this study did not specifically address cancer risk, the results suggest that long-term supplementation with DIM or I3C is not toxic and may up-regulate key xenobiotic metabolizing enzymes to reduce exposure to potentially carcinogenic compounds.

Table 11. Summary of animal model studies examining indole-3-carbinol, 3,3′-diindolylmethane, and cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Leibelt et al.[183]	Sprague-Dawley rats, n=140 (n=70 of each sex) 3 or 12 months: • Control diet • 50 mg/kg/d I3C • 6.6 mg/kg/d DIM • 66 mg/kg/d DIM	Body and tissue weights Clinical blood chemistry Serum 25-hydroxyvitamin D_3 Serum testosterone (in males only) Bone density analysis via histology Liver microsome and colon lysate CYP concentrations via SDS-PAGE	No significant differences in body weights between diets. Rats fed I3C had higher liver somatic indices, which was statistically significant among male rats only ($p = 0.0023$). Males consuming the highest dose of DIM also had significantly increased liver somatic indices ($p = 0.042$). Serum AST was significantly decreased in male rats fed I3C or DIM ($p = 0.013$). Serum ALP significantly lower in male rats fed I3C ($p = 0.01$) or the highest dose of DIM ($p = 0.004$). Serum CK was significantly decreased in male rats consuming the highest dose of DIM ($p = 0.01$). Serum vitamin D levels were significantly increased after 12 months of I3C consumption by both male ($p = 0.0371$) and female ($p = 0.0031$) rats. There were no significant changes in serum testosterone or estradiol by diet. There were no significant effects of diet on histopathology or bone density. I3C supplementation for 12 months significantly increased total hepatic CYP content in males and females ($p <0.05$). Hepatic CYP1A1 and 1A2 were significantly increased in males fed I3C (82-fold, $p <0.001$) or the high-dose of DIM (16-fold, $p = 0.032$). DIM and I3C also significantly increased hepatic CYP1A1/1A2 in female rats (no p values given). Colon CYP1A1 was increased in both male and female rats fed I3C or DIM (no p values given). In female rats fed I3C or DIM, colon CYP3A2 was increased, while only I3C up-regulated CYP3A2 in male rats (no p values given).	Increase in liver mass corresponds to increased concentrations of CYP.
Chang et al.[184]	Male C57BL/6 mice, n=18 12 w: • Control diet • High-fat diet	Adipose tissue macrophage infiltration via immunohistochemistry Nitrite production, IL-	High-fat diet significantly increased macrophage infiltration compared to the control diet ($p = 0.001$), while the high-fat diet + I3C significantly decreased macrophage infiltration compared to the high-fat diet ($p = 0.004$). Treatment of co-cultured cells with at least 10 μM I3C	No direct measurement of cancer

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	(>55% kcal) • High-fat diet + I3C (5 mg/kg body weight, 3 times weekly, i.p. 3T3-L1 preadipocytes and primary adipocytes harvested from experimental mouse epididymal adipose tissue • With or without RAW 264.7 macrophage co-culture (106 cells) for 24 h • 0-100 μM I3C for 24 h	6 concentration, gene expression, and adipocyte differentiation in primary adipocytes from epididymal adipose tissue with or without macrophage co-culture	significantly decreased nitrate production (p for trend <0.001), iNOS expression (p for trend = 0.001), and IL-6 levels in adipocytes in a dose-dependent manner (p <0.05). Treatment of co-cultured cells with any amount of I3C significantly increased PPARγ expression (p <0.001). Treatment of preadipocytes with at least 10 μM I3C significantly decreased triglyceride accumulation (p <0.05).	
Krajka-Kuzniak et al.[185]	Male Wistar rats, n=121, n=6 per group: • Water, 20% ethanol:olive oil, or olive oil as control • Cabbage and sauerkraut juices (1.25 ml/kg body weight) for 4, 10, or 30 d • I3C (100 mg/kg body weight) for 4, 10, or 30 d • PEITC (100	Liver and kidney cytosolic NQO1 and GST activities via substrate concentrations Liver and kidney GST, NQO1, and Nrf2 protein levels via SDS-PAGE	Raw cabbage juice and sauerkraut juice for 4 d significantly increased kidney GST activity (p <0.05). Raw cabbage juice and sauerkraut juice for 10 d significantly increased kidney NQO1 activity (p <0.05). I3C significantly increased kidney NQO1 activities at all time points (p <0.05) but did not significantly affect GST activity. PEITC significantly increased kidney GST activity at 4 and 10 d and significantly increased NQO1 activity at all time points (p <0.05). Raw cabbage juice for 10 d or sauerkraut juice for 30 d significantly increased liver GST α and μ expression compared to control treatment (p <0.05). Raw cabbage juice for 30 d and sauerkraut juice for 4 d significantly increased liver GST μ and NQO1 expression, respectively (p <0.05).	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	mg/kg body weight) for 4, 10 or 30 d		I3C for 4 d, 10 d, and 30 d significantly increased liver GST μ and NQO1 expression (p <0.05). I3C for 10 and 30 d also significantly increased liver GST α expression (p <0.05). Similar results were seen with PEITC. Raw cabbage juice and sauerkraut juice for 30 d significantly decreased kidney GST θ (p <0.05). Raw cabbage juice for 30 d significantly increased liver nuclear Nrf2 (p <0.05). I3C and PEITC significantly increased nuclear Nrf2 at all time points (p <0.05).	
Qian et al.[186]	Female A/J mice, n=100 Through week 27 (post-initiation): • NNK (4 doses of 50 mg/kg twice weekly for first 5 weeks prior to I3C treatment) with or without 10 μmol/g diet I3C • Untreated mice as controls Through week 52 (progression): • NNK (4 doses of 50 mg/kg twice weekly for first 5 weeks prior to I3C treatment) with or without 10 μmol/g diet I3C • Untreated mice as controls	Histopathology of lung tissues Lung cell proliferation via Ki-67 staining Apoptotic protein expression via kit Receptor tyrosine kinase/PI3K/Akt signaling protein expression via Western blot	Mice treated with I3C during post-initiation period had 51% fewer lung tumors than controls (p <0.0001), while I3C treatment during progression did not reduce tumor multiplicity. I3C treatment during both post-initiation and progression significantly increased the proportion of lung tumors fewer than 2 mm in diameter and significantly decreased the proportion of tumors greater than 2 mm in diameter compared to mice treated with NNK alone (p <0.05). I3C treatment during post-initiation significantly decreased the multiplicity of all histopathological lesions compared to NNK-treated controls (p <0.05). I3C treatment during progression significantly increased the proportion of hyperplastic foci and adenoma while significantly decreasing the proportion of adenoma with dysplasia and adenocarcinoma of the lung (p <0.05). I3C treatment significantly decreased proliferation in hyperplastic foci, adenoma, and adenoma with dysplasia histological subtypes of lung cells (p <0.05). I3C treatment following NNK treatment decreased p-Akt and survivin expression, while increasing PARP-cleavage, thereby enhancing apoptosis. I3C treatment also increased p-ERK levels, but it is not known what effect this may have on the cell cycle. Activation of 9 receptor tyrosine kinases was increased	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			with I3C treatment, while activation of 1 was decreased. I3C treatment increased the activation of 3 intracellular serine/threonine/tyrosine kinases, including p53, while the activation 8 of these proteins was decreased. I3C treatment up-regulated pro-apoptotic proteins while down-regulating anti-apoptotic proteins.	
Sepkovic et al.[187]	Female K14-HPV16 human cervical cancer mouse model, transgenic n=75, wild-type n=90, all mice implanted with E_2 pellets (0.25 mg/90 d throughout study) • Standard diet without or with 500, 1000, 1500, 2000, or 2500 ppm DIM	Histopathology of uterus and cervix Urinary DIM concentration via GC-MS	There was a significant increase in cervical intraepithelial neoplasia with increasing DIM (p <0.0001) in transgenic but not wild-type mice. 1000 ppm DIM was identified as the minimum effective dose (p = 0.0123). No cancers were documented at 1000 ppm DIM or higher. In both genotypes of mice, there was a significant positive increase in urinary DIM with increasing DIM (p <0.05).	Transgenic mice are homozygous for oncogene affected by HPV. 1000 ppm DIM is comparable to women taking 200 or 400 mg I3C twice daily for 4 w.

Abbreviations:
AST = aspartate aminotransferase
ALP = alkaline phosphatase
CK = creatinine kinase
PEITC = phenethyl isothiocyanate
NQO1 = NAD(P)H:quinine oxidoreductase 1
GST = glutathione *S*-transferase
Nrf2 = NF-E2-related transcription factor
NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PARP = poly ADP ribose polymerase
E_2 = estradiol
HPV = human papilloma virus

Krajka-Kuzniak et al.[185] compared the effects of cabbage and sauerkraut juices versus pure I3C or PEITC on xenobiotic metabolism in male Wistar rats over a four-, ten-, or 30-day period. The authors demonstrated that cabbage and sauerkraut juices as well as PEITC significantly increased kidney GST activity ($p < 0.05$), while all treatments significantly increased kidney NAD(P)H:quinine oxidoreductase 1 (NQO1) activity and liver GST and NQO1 expression ($p < 0.05$). While both cabbage and sauerkraut juices significantly decreased kidney GST θ expression following 30 days of supplementation ($p < 0.05$), this did not result in a decrease in overall GST activity, and the researchers cite evidence that GST θ may activate halogenated compounds to confer an increased risk of kidney cancer; therefore, a decrease in GST θ may be interpreted as chemopreventive. Lastly, I3C and PEITC at all time points and cabbage juice following 30 days of exposure significantly increased the nuclear translocation of Nrf2 ($p < 0.05$), indicating up-regulation of phase II metabolism as well as intracellular antioxidant systems.

In a mouse model of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung cancer, Qian et al.[186] distinguished the chemopreventive effects of I3C when administered during the post-initiation versus progression phases of lung cancer. Mice that received I3C during the post-initiation period (following five-week NNK treatment through week 27 of the experiment) showed a 51% reduction in the number of lung tumors compared to control animals not treated with I3C ($p < 0.0001$). I3C treatment during the progression period (from week 27 through 52) did not significantly reduce the number of lung tumors compared to controls. However, I3C treatment during either period significantly increased the number of smaller lung tumors (two mm or less

in diameter) and significantly decreased the number of larger lung tumors (greater than two mm in diameter) compared to control mice treated with NNK alone (p <0.05). Histopathological examination of the lung tissues showed that I3C treatment during post-initiation and progression significantly decreased all histopathological lesions and hyperplastic foci and adenoma, respectively, supporting the reduction in lung cancer transformation and progression with I3C treatment compared to controls (p <0.05). Regarding the mechanisms by which I3C decreases lung cancer severity, the researchers reported that I3C treatment significantly decreased lung cell proliferation among the histopathological lesions (p <0.05) while an important pro-apoptotic marker (PARP-cleavage) was up-regulated and two anti-apoptotic markers (p-Akt and survivin expression) were down-regulated. This study was also novel in its use of a proteome profiler array to identify the effects of I3C on receptor tyrosine kinases and serine/threonine/tyrosine kinases, which are involved in intracellular signal transduction; I3C treatment increased the expression of several pro-apoptotic proteins, including p53, while down-regulating anti-apoptotic proteins, such as Bcl-2, HIF-1α, and p-β-catenin.

In a mouse model of human cervical cancer, Sepkovic et al.[187] examined the effects of various concentrations of DIM on cervical histology in female mice homozygous or heterozygous for a specific oncogene affected by human papillomavirus-16 (HPV16), an important risk factor for cervical cancer. The authors concluded that mice homozygous for the oncogene were at a significantly decreased risk for cervical intraepithelial neoplasia (CIN, a precancerous lesion) with increasing amounts of DIM (p for trend <0.0001) and that the minimum effective dose of DIM was 1000 ppm (p =

0.0123), which is equivalent to a human dose of 400-800 mg I3C daily. No cervical or uterine cancers were identified in mice given 1000 ppm DIM or greater. In mice heterozygous for the oncogene, there was no significant effect of DIM in reducing CIN. The results from this study suggest that DIM is effective at reducing cervical cancer risk in individuals at high risk (homozygotes) while it does not have an appreciable effect on cervical histology in low risk individuals (heterozygotes), but confirmation in human beings is needed.

Finally, Chang et al.[184] reported the effects of a high-fat diet with or without I3C on inflammatory markers in adipocytes. I3C treatment significantly reduced the infiltration of macrophages into adipose tissue compared to a high-fat diet without I3C ($p = 0.004$). In the accompanying cell culture experiments, I3C treatment significantly decreased nitrate production (p for trend <0.001), inducible nitric oxide synthase (iNOS) expression (p for trend $= 0.001$), and IL-6 expression dose-dependently ($p <0.05$). Additionally, I3C treatment significantly reduced triglyceride accumulation in preadipocytes ($p <0.05$) and increased PPARγ expression in adipose tissue co-cultured with macrophages ($p <0.001$). Although this study did not specifically address cancer, the effects of I3C in decreasing inflammatory markers and increasing PPARγ expression are similar to those of n-3 fatty acids (see Chapter 1), and the relationship between these mechanisms and the risk of human cancers warrants further study.

Human observational study

Table 12 includes the sole observational study conducted on glucobrassicins and xenobiotic metabolism with regard to lung cancer risk by Hecht et al.[188]. These researchers explored the correlations between self-reported dietary intake of cruciferous vegetables, total ITC, and glucobrassicin with urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl-β-*O*-D-glucosiduronic acid (NNAL-Gluc) excretion among healthy Singapore Chinese smokers. Additionally, the levels of glucosinolates in cruciferous vegetables purchased in Singapore markets were confirmed with HPLC. Within these vegetables, glucobrassicins were the predominant glucosinolates and were present in especially high proportions in

Table 12. Summary of observational human study examining indole-3-carbinol, 3,3′-diindolylmethane, and cancer.

Reference	Study Design	Outcome Measurement(s)	Results
Hecht et al.[188]	Cross-sectional analysis of population-base prospective cohort Healthy smoking men and women, n=84, 45-74 y	Baseline diet via food-frequency questionnaire Smoking habits via in-person interview Glucosinolate content of cruciferous vegetables purchased in Singapore markets via HPLC Urinary NNAL and NNAL-Gluc via GC	Glucobrassicins were the predominant glucosinolates in 7 of 9 vegetables studied, representing 91% of glucosinolates in broccoli and 93% in cauliflower. There were significant correlations between number of cigarettes smoked daily and NNAL excretion ($p \leq 0.01$). Dietary glucobrassicins, dietary ITC, and total cruciferous vegetable intakes were significantly correlated with one another ($R^2 \geq 0.88$, $p < 0.0001$). There was a significant inverse association between glucobrassicin intake and free ($p = 0.01$) and total NNAL ($p = 0.03$). This association was borderline significant with NNAL-Gluc ($p = 0.08$). Data were not shown for total dietary ITC or cruciferous vegetable intake and urinary NNAL but stated to be similar to glucobrassicin.

Abbreviations:
NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc = 4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl-β-*O*-D-glucosiduronic acid

broccoli and cauliflower. These values were used to compute the mean daily glucobrassicin intake for each study subject, which demonstrated a significant inverse association with urinary free ($p = 0.01$) and total ($p = 0.03$) NNAL. These results suggested that dietary glucobrassicin enhances oxidative metabolism of NNK, likely through the actions of I3C and/or DIM.

However, the almost complete absence of epidemiological studies evaluating I3C, DIM, and cancer risk presents several challenges for their establishment as chemopreventive compounds. Unlike cell culture studies on this topic, which are abundant, epidemiological studies can provide valuable information as to the potential chemopreventive effects of DIM and I3C from typical dietary intakes of cruciferous vegetables. The large sample sizes required for such studies would also reduce inter-individual variability due to genetic differences and plant composition. Finally, consistency between experimental results and associations measured in free-living populations is one of the Bradford-Hill criteria to establish causation, and such evidence is necessary to establish specific public health recommendations regarding cruciferous vegetable intake and cancer risk[189].

Human pharmacokinetic studies

Given the interest in I3C or DIM therapy as potential chemopreventive agents, two human studies have established the pharmacokinetic as well as adverse event profiles of these compounds (Table 13). As part of a randomized placebo-controlled trial[190], Sepkovic et al.[191] measured urinary DIM in a subset of women with CIN and

Table 13. Summary of human intervention studies examining indole-3-carbinol and 3,3′-diindolylmethane pharmacokinetics.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
Sepkovic et al.[191]	Female patients with CIN II-III, n=30 Man of unknown health status	Pharmacokinetic evaluation from previous study by Bell et al.[190] I3C daily for 12 w: • Placebo • 200 mg • 400 mg For urinary elimination study, one man took 150 mg DIM twice daily, and urine was collected over 18 h.	Urinary DIM via GC-MS Urinary 2-OHE_1 and 16α-OHE_1	Women treated with either amount of I3C showed a significant increase in urinary 2-OHE_1:16α-OHE_1 compared to women treated with placebo (p <0.03). After 4 w of treatment, women receving 200 mg I3C daily (n=5) had mean urinary DIM of 12.1 ± 2.5 μg/mg creatinine and women receiving 400 mg I3C (n=5) had mean urinary DIM of 15.6 ± 22.2 μg/mg, which were not statistically different. Half-elimination time of DIM for individual was 3.5 h.	
Reed et al.[192]	Healthy non-smoking women, n=24, 23-58 y Subset of 14 women at increased risk of breast cancer (high Gail score or family history) used for pharmacokinetic study	For pharmacokinetic study, blood collected at 1, 2, 4, 8, 12, and 24 h following: • 400 mg I3C • 600 mg I3C • 800 mg I3C • 1000 mg I3C • 1200 mg I3C For single-dose study, 400 mg I3C twice daily for 4 w.	Adverse events via self-report Plasma DIM via HPLC-MS	Gastrointestinal distress was the main adverse event reported at 800 and 1200 mg I3C. Gastrointestinal symptoms were present in 25% of women consuming at least 600 mg I3C. Additional I3C condensation products (indolo-[3,2-*b*]-carbazole, trimers, and hydroxylated DIM were not detected. I3C was not detected in plasma samples at any time points. Change in plasma DIM is not linear between 600 and 1000 mg I3C. The maximum concentration of DIM is achieved between 2 and 3 hours following supplementation, while the maximum concentration following chronic I3C	6 subjects had highest DIM values in pre-dose plasma but values for 4 subjects dropped below detection at 12 h. This may be due to improperly timed supplementation on the part of the subjects. I3C may have been detected if first blood sampling had been <1 h; the half-life of this compound in mice was previously shown to be 0.25 h. Also, the

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
				supplementation occurs at 1.3 h. The half-life of DIM was between 2 and 5.7 h following the individual doses; the half-life of DIM following chronic I3C supplementation was 6 h.	gastric pH in rodents is higher than in humans. There was substantial variation in plasma DIM pharmacokinetics between individuals.

Abbreviations:
CIN = cervical intraepithelial neoplasia
2-OHE_1 = 2-hydroxyestrone
16α-OHE_1 = 16α –hydroxyestrone

concluded that the excretion of DIM was not statistically different between women consuming 200 and 400 mg I3C per day for four weeks. From 200 mg I3C, the mean ± standard deviation of urinary DIM was 12.1 ± 2.5 μg/mg creatinine, and from 400 mg I3C, the corresponding values were 15.6 ± 22.2 μg/mg creatinine. Although these values were not statistically different, there is substantial variation, particularly within women receiving the higher dose of I3C. This deviation suggests a high degree of inter-individual variation regarding absorption, metabolism, and excretion of I3C and DIM. Nevertheless, the researchers measured urinary excretion of DIM within one man over 18 hours following a single dose of 150 mg DIM and calculated the half-elimination time (a corollary to half-life) as 3.5 hours, indicating relatively rapid excretion of DIM. An important limitation of these experiments was the very small sample sizes (n=5 for each I3C treatment and n=1 for the DIM treatment), resulting in large variation and preventing the extrapolation of results to other populations. Another major limitation of this study was the use during sample preparation of a deconjugation enzyme derived from *Helix pomatia*, which has been shown to be contaminated with several phytonutrients, including DIM (see Appendix).

In a slightly larger study, Reed et al.[192] calculated the pharmacokinetics of plasma DIM in non-smoking women at an increased risk of breast cancer (n=14) given 400, 600, 800, 1000, or 1200 mg of I3C at one time or 800 mg I3C daily for four weeks. The women also reported any adverse events experienced with taking I3C, of which gastrointestinal distress was the most common. Such symptoms were present in 25% of all women taking at least 600 mg I3C. Blood was first collected at one hour following

the final dose of I3C, at which time no plasma I3C was detected. The researchers also did not detect the additional condensation products hydroxylated-DIM, trimers, or ICZ. Similar to the results of Sepkovic et al.[191], the peak plasma DIM concentration appeared between two and three hours following single I3C supplementation, and the half-life of plasma DIM was between two and 5.7 hours. In the women that chronically supplemented with I3C over four weeks, the peak concentration of plasma DIM occurred at 1.3 hours, and the half-life of plasma DIM was six hours. Again, these results demonstrate the rapid metabolism of I3C to form DIM, which is excreted with a half-life between one and six hours. An intriguing result from this study showed that the change in plasma DIM was not linear between the adminstration of 600 and 1200 mg I3C, while the peak concentration of plasma DIM with 1200 mg I3C was not greater than with 1000 mg I3C. This information suggests that saturation of one or more steps of I3C absorption and metabolism and DIM formation occurs at intakes of I3C between 600 and 800 mg per day. Together with the increased frequency of adverse events at 600 mg or greater I3C, this information suggests that doses of I3C in excess of 600 mg may not be well-tolerated or more effective than lower doses.

Although the work by Reed et al.[192] was conducted in a greater number of subjects, there was considerable variation in DIM pharmacokinetics between subjects, as in the work by Sepkovic et al.[191]. Another troubling observation by Reed et al.[192] was the presence of the highest concentration of DIM in the plasma of six of fourteen subjects participating in the chronic supplementation study prior to the administration of I3C for pharmacokinetic measurements. This fact was attributed to the non-compliance

of the subjects regarding timing of their previous dose of I3C, but this may not fully explain the high baseline levels in such a large proportion of subjects. Although the authors did not use the *H. pomatia* enzyme shown to be contaminated with DIM, there may be other sources of contamination throughout their assay, poor specificity of their equipment or technique, or additional metabolic characteristics of these subjects for which the authors did not account (i.e. genotypes of metabolizing enzymes, variations in gastric pH, or the use of medications). Plasma DIM dropped below the limit of detection within twelve hours for most of the subjects, reducing the evidence for contamination. Nevertheless, these issues need to be satisfactorily addressed to establish a sensitive and specific biomarker of I3C exposure as well as the pharmockinetics of I3C and DIM.

Human intervention studies

To date, most human intervention trials of DIM or I3C, as summarized in Table 14, have focused on hormone-dependent cancer biomarkers among women. However, an early experiment conducted by Taioli et al.[165] evaluated the response to NNK metabolism among healthy female smokers given 400 mg I3C daily. Compared to baseline values, I3C supplementation significantly decreased free (23.4%, $p = 0.016$) and total (10.9%, $p = 0.023$) NNAL while significantly increasing NNAL-Gluc:NNAL by nearly 40% ($p = 0.046$). These results implicate I3C—or more likely, DIM—in up-regulating CYP1A2 metabolism of NNK via α-hydroxylation rather than reduction to NNAL; the relative increase in glucuronidated NNAL also suggests an up-regulation of UGT enzymes. Such changes in these enzyme systems are postulated to reduce the

Table 14. Summary of human intervention studies examining indole-3-carbinol, 3,3′-diindolylmethane, and cancer.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
Taioli et al.[165]	Healthy female smokers, n=13, 24-60 y	400 mg/d I3C for 5 d	Urinary NNAL and NNAL-Gluc via GC Urinary cotinine via radioimmunoassay Urinary creatinine via clinical chemistry kit	Supplementation with I3C significantly decreased urinary free NNAL by 23.4% (p = 0.016) and total NNAL by 10.9% (p = 0.023) compared to baseline. There was also a significant 39.9% increase in NNAL-Gluc:NNAL compared to baseline (p = 0.046). There were no significant changes in urinary cotinine.	These results suggest I3C induced CYP1A2 and possibly UGT, which may also have been saturated. In contrast, previous studies have shown that PEITC inhibits CYP1A2.
Bell et al.[190]	Female patients with CIN II-III, n=30	I3C daily for 12 w: • Placebo • 200 mg • 400 mg	CIN via cervical biopsy HPV status via PCR Urinary 2-OHE_1 and 16α-OHE_1	In women treated with either amount of I3C, there were significant reductions in CIN progression (200 mg: relative risk (RR) = 0.50, 95% CI 0.25—0.99, p = 0.023; 400mg: RR = 0.55, 95% CI 0.31—0.99, p = 0.032). Urinary 2-OHE_1:16α-OHE_1 increased in a dose-dependent manner but did not reach statistical significance.	
Sepkovic et al.[191]	Female patients with CIN II-III, n=30	I3C daily for 12 w: • Placebo • 200 mg • 400 mg	Urinary DIM via GC-MS Urinary 2-OHE_1 and 16α-OHE_1	Subgroup of women (n=10) treated with either amount of I3C showed a significant increase in urinary 2-OHE_1:16α-OHE_1 compared to women treated with placebo (n=7) (p <0.03).	
Dalessandri et al.[193]	Non-smoking, postmenopausal women in Marin County, CA diagnosed with early-stage (0-2) breast cancer, n=19, 55-69 y	Randomized, double-blind control trial of: • Placebo daily for 30 d • BioResponse DIM 108 mg/d for 30 d	Urinary E_1, E_2, and E_3 concentrations via GC-MS Urinary 2-OHE_1 and 16α-OHE_1 via immunoassay Urinary cortisol and 6β-OHC via ELISA Urinary DIM via	DIM supplementation significantly increased 2-OHE_1 compared to placebo (p = 0.02). 2-OHE_1:16α-OHE_1 increased by 47% due to DIM supplementation (p = 0.059). There were no significant differences in urinary and 16α-OHE_1, E_1, E_2, and E_3 in DIM vs. control. DIM supplementation significantly	Results adjusted for baseline DIM. DIM detected in urine of placebo group and at baseline possibly due to use of contaminated enzyme. DIM believed to have less effect on CYP3A4

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
			spectrophotometry	increased urinary DIM (p = 0.045) and cortisol (p = 0.039) relative to control.	than I3C, explaining non-significant reduction in 6β-OHC:cortisol. Increase in cortisol suggests DIM may stimulate adrenal glands.
Reed et al.[194]	Healthy, non-smoking women women at increased risk of breast cancer (high Gail score or family history), n=17, 15-65 y	Following 4 w run-in with placebo, all subjects consumed 400 mg I3C daily for 4 w, then 800 mg/d for 4 w.	Adverse events via self-report Plasma 2-OHE_1, 16α-OHE_1, and 6β-OHC via immunoassay Serum estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, sex hormone binding globulin, thyroid-stimulating hormone, and insulin-like growth factor binding protein-1 and -3 CYP1A2, NAT-2, xanthine oxidase, and FMO3 activities via urinary caffeine excretion CYP2D6 activity via urinary dextromethorphan excretion Lymphocyte cytosolic	There were no serious adverse events reported. There were no significant changes in serum hormone profiles. 800 mg I3C treatment significantly increased CYP1A2 activity 5-fold compared to placebo (p <0.0001). Among fast acetylators (determined by baseline NAT-2 activity), 800 mg I3C treatment significantly decreased NAT-2 activity by 18% (p = 0.012). There was no significant change among slow acetylators. 800 mg I3C treatment significantly decreased FMO3 activity by 60% compared to placebo (p = 0.02). There were no significant changes in xanthine oxidase, CYP2D6, CYP3A (determined by urinary 6β-OHC:cortisol) activities with I3C supplementation. Both doses of I3C significantly increased 2-OHE_1:16α-OHE_1 to the same extent relative to control (~66%, p <0.0001). 800 mg I3C treatment significantly increased lymphocytic GST activity by 69% compared to placebo (p = 0.002).	

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
			NQO1 and GST activities via spectrophotmetry	There were no significant changes in NQO1 activity.	
Connor et al.[195]	Healthy women, n=33	200 mg I3C twice daily for 8 w	Urinary 2-OHE_1 and 16α-OHE_1 via Estramet system	There were no significant changes in urinary estrone ratio among morbidly obese women. There was decreased response to I3C with increasing BMI. The estrone ratio increased to a greater degree (71%) in women with low BMI compared to those with high BMI (p = 0.09). 16α-OHE_1 significantly increased among obese women with I3C treatment. However, significantly fewer women with low BMI showed less of an increase compared to women with high BMI (p = 0.04), and the difference in urinary 16α-OHE_1 was significantly different between these two groups (p = 0.04).	Abstract of pilot study Low BMI = healthy or overweight High BMI = obese or morbidly obese
Hauder et al.[196]	Healthy male non-smokers, n= 76, 50-82 y	Subjects randomly assigned to consume daily for 4 w: • 200 g/d regular blanched broccoli • 200 g/d selenium-fertilized broccoli • Placebo	Serum selenium via atomic absorption spectroscopy Plasma and urine glucoraphanin (sulforaphane, sulforaphane-NAC, -glutathione, -Cys-Gly, and –Cys) and glucobrassicin (DIM, I3C-acetonitrile, -aldehyde, -carboxylic acid, and ascorbigen) metabolites via LC-MS/MS	Selenium fertilizer increased total glucosinolate content of broccoli by 20%. Consumption of selenium-fertilized broccoli, which contained 25 times more selenium than regular broccoli, significantly increased serum selenium concentrations compared to regular broccoli (p <0.001). Regular broccoli consumption significantly increaed urinary and plasma SFN-Cys-Gly, SFN-Cys, and SFN-NAC compared to baseline (p <0.05). Selenium-fertilized broccoli consumption significantly increased urinary and plasma SFN and SFN-NAC, urinary SFN-Cys, and	Free I3C, I3C-acetonitrile, and DIM were not detected in any samples. SFN-Cys was present in baseline urine samples from all groups and may be a long-lived metabolite not completely excreted from 1 w of abstaining from cruciferous vegetables prior to study. I3C-carboxylic acid

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
		capsules		plasma SFN-Cys-Gly (p <0.05). The placebo supplement significantly increased plasma SFN-Cys and decreased urinary SFN, SFN-Cys-Gly, and SFN-NAC (p <0.05). Regular broccoli consumption significantly increased plasma and urinary ascorbigen, plasma I3C-carboxylic acid, and urinary 13C-aldehyde (p <0.05). Selenium-fertilized broccoli consumption significantly increased plasma and urinary I3C-carboxylic acid and ascorbigen and urinary I3C-aldehyde (p <0.05).	and -aldehyde have been shown as metabolites of tryptophan.

Abbreviations:
NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc = 4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl-β-*O*-D-glucosiduronic acid
CIN = cervical intraepithelial neoplasia
2-OHE_1 = 2-hydroxyestrone
16α-OHE_1 = 16α –hydroxyestrone
6β-OHC = 6β-hydroxycortisol
NAT-2 = *N*-acetyltransferase-2
FMO3 = flavin monooxygenase-3

exposure of an organism to carcinogens from cigarette smoking, thereby reducing cancer risk.

Bell et al.[190] conducted a randomized placebo-controlled trial of I3C in 30 women with stage II or III CIN and concluded that 200 or 400 mg of I3C daily for twelve weeks significantly decreased the progression of CIN (200 mg: relative risk (RR) = 0.50, 95% confidence interval (CI) 0.25—0.99, $p = 0.023$; 400 mg: RR = 0.55, 95% CI 0.31—0.99, $p = 0.032$). In this analysis, the increase in urinary 2-hydroxyestrone (2-OHE_1):16α-hydroxyestrone (16α-OHE_1) with I3C supplementation—considered protective against tumor growth—did not reach statistical significance. However, the previously mentioned subset analysis from this trial by Sepkovic et al.[191] concluded there was a significant increase in urinary 2-OHE_1:16α-OHE_1 among ten women taking 200 or 400 mg I3C daily for twelve weeks compared to women consuming a placebo ($p < 0.03$).

In a placebo-controlled study of 19 women with early-stage breast cancer, Dalessandri et al.[193] evaluated the effects of a DIM supplement (108 mg/d for 30 d) on urinary estrogen metabolites. Among women consuming the DIM supplement, urinary 2-OHE_1 significantly increased compared to among women consuming the placebo ($p = 0.03$). There was also a 47% increase in urinary 2-OHE_1:16α-OHE_1 among women taking DIM ($p = 0.059$). However, there were no significant differences in urinary concentrations of other estrogen metabolites. The change in urinary 2-OHE_1 suggests that DIM up-regulates CYP1A1, while the non-significant decrease in urinary 6β-hydroxycortisol (6β-OHC):cortisol ($p = 0.16$) suggests that DIM does not appreciably

affect CYP3A4. The researchers noted that baseline DIM was detected in women in the placebo group and adjusted all their results for baseline DIM values; again, this is likely due to the use of a contaminated enzyme during sample processing.

Reed et al.[194] conducted a similar pilot study among 17 women at increased risk of breast cancer to monitor adverse events and effects on serum hormones and xenobiotic metabolism activity via plasma concentrations and urinary excretion of metabolites. Following a four-week run-in period during which all subjects consumed a placebo, the women consumed 400 mg of I3C daily for four weeks followed by 800 mg of I3C daily for four weeks. No serious adverse events were reported, and there were no significant changes in the serum hormone profiles. The highest dose of I3C significantly increased CYP1A2 activity by five-fold as measured by caffeine excretion (p <0.0001 versus placebo). The researchers elected to divide the subjects based upon baseline *N*-acetyltransferase-2 (NAT-2) activity; among women with high NAT-2 activity, 800 mg I3C significantly decreased NAT-2 activity by 18% (p = 0.012). There was no such effect among women with low NAT-2 activity at baseline. In addition, the highest dose of I3C significantly decreased flavin monooxygenase-3 (FMO3) activity by 60% (p = 0.02), but further research is required to confirm this result. The investigators also measured GST and NQO1 activity in lymphocytes extracted from the subjects and concluded that GST significantly increased by 69% with 800 mg I3C compared to placebo (p = 0.002). There were no significant changes in NQO1, xanthine oxidase, CYP2D6, or CYP3A activities with I3C supplementation. Similar to other studies, the researchers measured a significant 66% increase in plasma 2-OHE_1:16α-OHE_1 with

either dose of I3C compared to placebo (p <0.0001). These results provide further evidence that I3C and DIM may decrease breast cancer risk by up-regulating CYP1A2 activity to promote the metabolism of estrogen to 2-OHE_1, which is considered less tumorigenic than 16α-OHE_1. The effect of I3C on other enzymes such as FMO3 and GST require further study but may also reduce exposure to compounds that potentially cause or promote cancer.

In an abstract for a pilot study, Connor et al.[195] reported the effects of 200 mg I3C on urinary 2-OHE_1:16α-OHE_1 in 33 healthy women of various body sizes. This ratio increased to a greater degree among healthy or overweight women compared to obese or morbidly obese women (71%, p = 0.09). Notably, the concentration of 16α-OHE_1 significantly increased among obese women consuming I3C, but this increase was significantly lower among healthy or overweight women (p = 0.04). The authors noted an inverse relationship between the effect of I3C and body weight but did not indicate if this was a statistically significant trend. Nevertheless, these results suggest that the ability of DIM or I3C to favorably alter estrogen metabolism is likely influenced by body weight, an important consideration for further clinical trials and epidemiological studies.

Finally, in a validation study not directly related to cancer, Hauder et al.[196] quantified the urinary and plasma metabolites of glucoraphanin and glucobrassicin in 76 healthy men consuming placebo capsules, 200 g regular blanched broccoli, or 200 g selenium-fertilized broccoli daily for four weeks. The researchers also measured the glucosinolate content of the broccoli, which increased by 20% with the selenium-enriched fertilizer. As expected, consumption of the selenium-fertilized broccoli

significantly increased serum selenium compared to consumption of the regular broccoli (p <0.001). SFN metabolites significantly increased among all groups (including the placebo) (p <0.05), suggesting these compounds may be relatively persistent in the body. Broccoli consumption also significantly increased ascorbigen (formed from the reaction of I3C with ascorbic acid), I3CA, and I3A. Although the LC-MS/MS techniques were validated with high sensitivity and specificity, the authors were not able to detect free I3C, I3C-acetonitrile, or DIM in any plasma or urine samples. They suggested that the I3C metabolites detected (I3CA and I3A) may have been generated from tryptophan metabolism, and the glucobrassicin content of the broccoli remained relatively low (111 and 107 μg/g fresh weight in regular and selenium-fertilized, respectively), which may partially explain their null results. Additionally, urine samples were collected one day after discontinuing the broccoli treatment; given the very short half-life of DIM (less than six hours), it is not surprising that these investigators were not able to detect urinary DIM from the broccoli consumption 24 hours prior.

While several human intervention studies have shown an improvement in estrogen metabolism with DIM or I3C supplementation, more research into this field is required. Only one study reviewed concluded that I3C reduced the progression of cervical neoplasia; quantification of sensitive and specific cancer-related endpoints in large numbers of subjects would provide valuable evidence for I3C and DIM as chemopreventive agents. Studies exploring additional cancer sites, especially lung and prostate cancer, are also needed to confirm the results from *in vitro* and in animal studies. However, this field remains in relative infancy—especially in the absence of a sensitive

and specific biomarker of I3C exposure—and a search of the National Institutes of Health clinical trials registry show a total of eleven studies of I3C or DIM and prostate, breast, cervical, or all cancers either completed or currently recruiting subjects.

Conclusions

The studies reviewed above establish I3C and—more promisingly—DIM as potentially effective chemopreventive and chemotherapeutic agents. *In vitro* experiments demonstrated that these compounds can trigger cancer cell cycle arrest and apoptosis by altering gene transcription. Animal models confirmed these effects in the reduction of lung tumor size and multiplicity and cervical neoplasia in the presence of HPV16 as well as up-regulation of phase I and II xenobiotic metabolism enzymes. The prospective cohort study suggested that I3C exposure favorably altered metabolism of a tobacco-specific carcinogen to potentially reduce the risk of tobacco-related cancers. Finally, the clinical trials conducted to date have shown that DIM or I3C supplementation beneficially altered estrogen metabolism to possibly reduce the risk of estrogen-associated cancers. These data also proved that DIM and I3C up-regulate phase I and II metabolism in healthy adults, which may reduce the risk of multiple cancers.

However, a significant limitation of the research regarding I3C exposure and cancer risk is the lack of observational data in human populations. I3C cannot typically be detected in tissues, and its most prevalent metabolite, DIM, has a relatively short half-life of less than six hours. This creates a challenging scenario for objective assessment of I3C exposure in free-living populations. The development of a highly sensitive, accurate,

and reliable biomarker of I3C exposure is the first step to accurately characterize the relationship between I3C and cancer risk in healthy populations. Ideally, this biomarker could be broadly applied and measured over time in free-living populations to fully ascertain the protection imparted by I3C or DIM against human cancers.

Chapter IV: Validation of an assay for the measurement of 3,3'-diindolylmethane as a marker for indole-3-carbinol exposure in free-living subjects

Introduction

Consumption of cruciferous vegetables in the genus *Brassica*, including broccoli, cabbage, cauliflower, and Brussels sprouts, has been inversely associated with the risk of several human cancers including lung and colorectal cancers[156, 159]. Glucobrassicin, a glucosinolate found in *Brassica* vegetables, undergoes hydrolysis in the presence of myrosinase to form indole-3-carbinol (I3C), which has demonstrated chemopreventive properties *in vivo* but is relatively short-lived in humans[155, 186, 192, 197-199]. At gastric pH, I3C oligomerizes to produce several metabolites, the most prevalent of which is 3,3'-diindolylmethane (DIM)[191, 192, 194]. Although urinary DIM has been measured in individuals participating in clinical trials of DIM and/or I3C supplementation, an accurate and reliable technique for the quantification of DIM as an objective marker of glucobrassicin intake in large observational studies is lacking[191, 193]. The objective of these experiments was to validate a sensitive technique to quantify urinary DIM using HPLC and mass spectrometry (MS).

Materials and Methods

Chemicals

All chemicals were HPLC, LC-MS, or Optima grade. DIM was purchased from LKT Labs (Saint Paul, MN). Indole, 4-methoxy indole, ammonium acetate, and *t*-butyl methyl ether were obtained from Sigma-Aldrich (St. Louis, MO). d_2-formaldehyde (isotopic purity 98%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). ^{14}C-formaldehyde was obtained from Moravek Biochemicals (Brea, CA).

Methanol, chloroform, hexane and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). β-Glucuronidase/arylsulfatase from *Helix pomatia* (cat. no. 10127698001) was purchased from Roche (Penzberg, Germany). β-Glucuronidase preparations from *Escherichia coli* (G8295) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate buffered saline 1X was purchased from Gibco by Life Technologies (Grand Island, NY). D_2-DIM and ^{14}C-DIM were synthesized as previously described[200].

Experiment 1: Comparison of solid-phase and liquid-liquid extractions

Control urine samples following abstinence from cruciferous vegetable intake were spiked with 0, 1.01, 2.02, 4.05, or 16.2 pmol DIM/mL, and all samples were analyzed in duplicate. Five replicates with 2.02 pmol DIM were included in each technique to analyze accuracy and precision. A flow-chart of the experimental design is shown in Figure 21. A 1 mL urine sample was placed in a 4 mL silanized vial (Chrom Tech, Apple Valley, MN) and 80.3 pmol each of d_2-DIM and 4-methoxy indole were added as internal standards. The pH was adjusted to 5 with the addition of 150 µL of 2.5M NaOAC (pH 5.8). 20 µL of β-glucuronidase/arylsulfatase (2000 units) were added to half of the samples, and the mixtures were incubated for 24 h at 37 ^{0}C. The samples were loaded on Strata-X-SPE cartridges (33µm polymeric reverse phase 200 mg/6 mL, Phenomenex, Torrance, CA) that were previously equilibrated using 6 mL of methanol followed by 6 mL of water. The cartridges were sequentially eluted with 3 mL of water, 3 mL of 10% methanol:water, and 3 mL of 30% which was collected in a 4 mL silanized

vial and dried under reduced pressure using a centrifugal vacuum evaporator (Savant SpeedVac) or via N_2 gas at ambient temperature. The residue was then transferred with 100 µL 30% chloroform:acetonitrile to a silanized autosampler GC vial with an infused 300 µL insert and dried again under vacuum. The final sample volumes were made up to 100 µL with 30% acetonitrile:10 mM ammonium acetate.

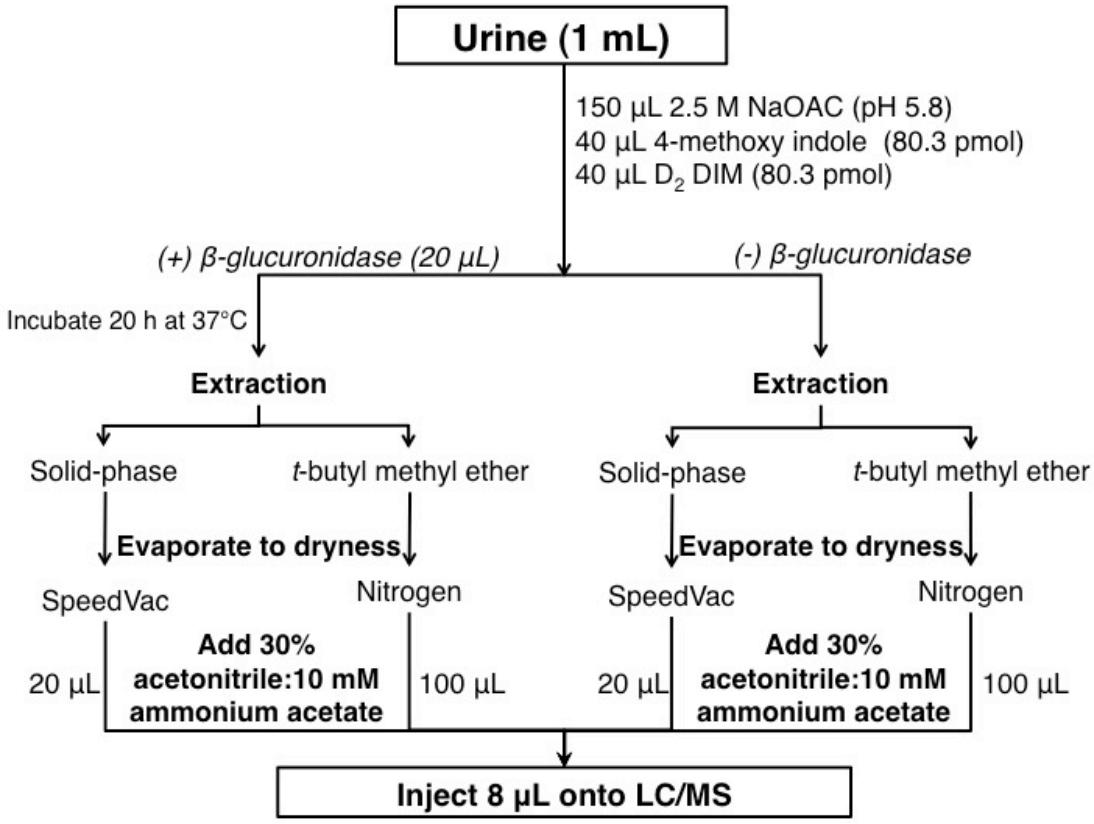


Figure 21. Design for validation Experiment 1.

Alternatively, each 1 mL sample with internal standards and sodium acetate were treated with or without 2000 U β-glucuronidase/arylsulfatase followed by liquid-liquid extraction with an equal volume of *t*-butyl methyl ether twice[196]. The pooled extracts were dried under vacuum or N_2 gas and transferred to 300 μL GC vials as above. The final sample volumes were made up to 20 μL (vacuum evaporation) or 100 μL (N_2 evaporation) with 30% acetonitrile:10 mM ammonium acetate. Three replicates of control urine with 20 μL of ^{14}C-DIM were prepared with or without 2000 U β-glucuronidase/arylsulfatase and liquid-liquid extraction with vacuum or N_2 evaporation or solid-phase extraction with vacuum evaporation and analyzed for ^{14}C recovery via scintillation counting (Beckman Coulter LS6500 Scintillation Counter, Brea, CA).

Experiment 2: Validation of liquid-liquid extraction technique with physiological concentrations of DIM

This experiment utilized the liquid-liquid extraction followed by centrifugal vacuum evaporation outlined in Experiment 1; however, 20 pmol d_2-DIM were used as an internal standard rather than 80.3 pmol. Control urine samples (in duplicate) were spiked with 0, 2.5, 5.1, 10.1, 20.2, or 40.5 pmol DIM. No β-glucuronidase/arylsulfatase was used in the control urine samples.

To assess the technique in urine samples from subjects consuming cruciferous vegetables, 0.5 mL urine from 24-hour urine collection samples from three individuals consuming 50 g Brussels sprouts were analyzed in duplicate, with or without the addition of 1000 U β -glucuronidase/arylsulfatase and sodium acetate. The samples were prepared

identically to the control urine specimens. Additionally, five replicates of control urine spiked with 5.1 pmol DIM without β-glucuronidase/arylsulfatase treatment were analyzed for accuracy and precision using the liquid-liquid extraction and vacuum evaporation method with 20 pmol internal standard.

Experiment 3: Assessment of inter-day reproducibility and reduced internal standard concentration

The same technique as above was used with 10 pmol d_2-DIM rather than 20 pmol as an internal standard. Spot urine samples from ten subjects abstaining from cruciferous vegetables for five days and 24-hour urine samples from the same subjects after consuming 50 g Brussels sprouts were analyzed in duplicate with the addition of 2000 U β-glucuronidase/arylsulfatase, and analyses of five subjects were repeated on three days for the spot urines and two days for the Brussels sprouts urines to assess inter-day reliability of the technique. Five samples of each spot and Brussels sprouts urine were repeated over two days using a supported liquid-liquid extraction via 3 mL ChemElut cartridges (Agilent Technologies, Santa Clara, CA) eluted with hexane followed by *t*-butyl methyl ether. Finally, five Brussels sprouts urine samples were treated with 6000 U β-glucuronidase/arylsulfatase to compare quantification of DIM versus 2000 U β-glucuronidase/arylsulfatase.

A water blank treated with internal standard and β-glucuronidase/arylsulfatase was included with each day of assays, and upon observing DIM detected in these water blanks, four water blanks with 1000 U, 2000 U, 4000 U, or 6000 U β-

glucuronidase/arylsulfatase were analyzed for DIM contamination.

Experiment 4: Evaluate contamination of H. pomatia *β-glucuronidase/arylsulfatase with DIM*

One mL HPLC-grade water was treated in duplicate with 2000 U *H. pomatia* β-glucuronidase/arylsulfatase from two lots, 2000 U *E. coli* β-glucuronidase, or no enzyme were prepared as in Experiment 2. Additionally, 1 mL of water treated with Roche β-glucuronidase or *E. coli* β-glucuronidase/arylsulfatase was analyzed with or without the addition of d_2-DIM as an internal standard, and 1 mL of water without enzyme treatment was analyzed with the addition of 5 or 10 pmol d_2-DIM to quantify the contribution of DIM from the internal standard.

Experiment 5: Compare β-glucuronidase from H. pomatia *and* E. coli *to no enzyme treatment for DIM quantification and reliability*

This experiment compared the use of 2000 U semi-purified β-glucuronidase/arylsulfatase from *H. pomatia* to 2000 U *E. coli* β-glucuronidase or no enzyme treatment in two individuals abstaining from cruciferous vegetables (spot urine samples) or following the consumption of 50 g Brussels sprouts (24-hour urine samples). Water blanks were included with each method, and control 24-hour urine samples from an individual abstaining from cruciferous vegetables for seven days and the same individual following the consumption of 200 g Brussels sprouts were included as negative and positive controls, respectively. As in Experiment 3, baseline urine samples

were analyzed in duplicate on three days, and Brussels sprouts urine samples were analyzed in duplicate on two days (except for the positive control urines, which were analyzed on three days) to ascertain inter-day reproducibility of the technique. The only modification to the liquid-liquid extraction technique described above was the use of 5 pmol d_2-DIM as an internal standard rather than 10 pmol to minimize the contribution of unlabeled DIM from the internal standard.

Experiment 6: Validate final technique for urinary DIM quantification

Positive and negative control urine samples (1 mL) were treated with 2000 U *E. coli* β-glucuronidase and 5 pmol d_2-DIM and prepared as described previously. Five replicates of each sample were analyzed on two days to assess accuracy, reliability, and inter-day reproducibility of the technique. To validate the method in a range of physiological DIM concentrations, negative urine samples containing 20%, 40%, 60%, or 80% positive control urine were analyzed in duplicate. Finally, 1.25, 2.55, 5.05, 10.1, 20.25, or 30.35 pmol DIM were added to negative control urines, which were analyzed to further validate the final method.

Quantitation of Urinary DIM by Capillary LC/ESI-MS/MS-SRM

The analyses were carried out by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM) on a TSQ Quantum Discovery Max instrument (Thermo Fisher Scientific, Waltham, MA) in the positive ion mode with N_2 as the nebulizing and drying gas. MS

parameters were set as follows: spray voltage, 3.2 kV; sheath gas pressure, 25 (arbitrary units); capillary temperature, 250 °C; collision energy, 17 V; scan width, 0.05 amu; Q2 gas pressure 1.0 mTorr; source CID 9 V; and tube lens offset, 104 V. MS data were acquired and processed by Xcalibur software version 1.4 (Thermo Electron, Waltham, MA). Eight microliters of the sample were injected from an autosampler into an Agilent 1100 capillary HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a 5μm, 150 x 0.5 mm ZorbaxSB-C18 column (Agilent Technologies, Santa Clara, CA) eluted at 15 μl/min for the first 3 min then 10 μl/min with a gradient from 60% methanol in 15 mM ammonium acetate to 100% methanol in 8 min and held for additional 29 min. The mass transitions (parent to daughter) monitored were *m/z* 247→130 for DIM (retention time averaged 16.6 min) and *m/z* 249→132 for d_2-DIM (retention time averaged 16.5 min) as an internal standard. Quantitation was accomplished by comparing the MS peak area ratio of DIM to that of the deuterated standard with a calibration curve of the concentration of DIM versus the MS peak area ratio (DIM:internal standard). The assay limit of quantitation (LOQ) was 132 fmol.

Statistics

All statistics were computed with SAS v9.2 (SAS Institute, Inc., Cary, NC). Comparisons of urinary DIM before and after exposure to cruciferous vegetables and by technique were evaluated with paired t-tests for lognormal distributions. The change in DIM concentration with increasing amounts of Roche *H. pomatia* β-

glucuronidase/arylsulfatase was evaluated with linear regression. The level of significance was $\alpha < 0.05$.

Results

Experiment 1: Comparison of solid-phase and liquid-liquid extractions

Table 15 summarizes the results of the experiment comparing the previous solid-phase extraction and centrifugal vacuum evaporation technique to the *t*-butyl methyl ether extraction and N_2 evaporation technique for urinary DIM quantification. Overall recoveries of both internal standards (d_2-DIM and 4-methoxy indole) were low, averaging 5.68% and 1.26%, respectively. The *t*-butyl methyl ether extraction method improved recovery of both internal standards. Given the low recovery and poor accuracy and reliability of 4-methoxy indole as an internal standard, d_2-DIM was preferable as an internal standard and was used in all following methods.

Although the calibration curves shown in Figure 22 show the solid-phase extraction technique to be more accurate than the *t*-butyl methyl ether extraction technique according to R^2 values, the *t*-butyl methyl ether extraction coupled to evaporation via centrifugal evaporation showed the highest rates of d_2-DIM recovery (Table 16). From the additional precision and accuracy analyses shown in Table 17, the *t*-butyl methyl ether extraction technique with β-glucuronidase/arylsulfatase treatment had the best accuracy and reliability of the four techniques assessed. Finally, the ^{14}C recovery experiment demonstrated the best recovery of DIM with *t*-butyl methyl ether extraction followed by centrifugal vacuum evaporation (Table 18).

Table 15. Summary of Experiment 1 comparing solid-phase and liquid-liquid extraction techniques for urinary DIM quantification.

Sample preparation technique			Solid-phase extraction and centrifugal vacuum evaporation				Liquid-liquid extraction and N_2 evaporation			
			d_2-DIM as internal standard		4-methoxy indole as internal standard		*d_2*-DIM as internal standard		4-methoxy indole as internal standard	
Enzyme treatment	Sample*	DIM added (pmol)	% recovery	DIM (pmol/mL urine or water ± SE)	% recovery	DIM (pmol/mL urine or water ± SE)	% recovery	DIM (pmol/mL urine or water ± SE)	% recovery	DIM (pmol/mL urine or water± SE)
(-) β-glucuronidase/ arylsulfatase	Water	0	7.02%	<LOQ	5.16%	0.22 ± 0.15	22.65%	0.13 ± 0.06	2.34%	31.60 ± 25.42
	Control urine	0	2.18%	1.33 ± 0.03	0.36%	14.06 ± 2.36	9.19%	<LOQ	7.94%	0.40 ± 0.33
	Control urine	1	3.86%	1.55 ± 0.31	0.52%	18.45 ± 2.44	1.56%	1.94 ± 1.87	0.39%	44.73 ± 44.66
	Control urine	2	3.91%	2.82 ± 0.77	0.39%	42.41 ± 7.49	6.00%	1.00 ± 0.93	0.47%	86.81 ± 86.74
	Control urine	4	3.57%	2.99 ± 0.14	0.56%	38.99 ± 21.20	2.50%	1.21 ± 1.14	0.31%	59.64 ± 59.57
	Control urine	16.2	3.32%	10.73 ± 1.15	0.30%	178.42 ± 16.48	9.58%	5.69 ± 0.76	0.31%	731.75 ± 164.79
(+) β-glucuronidase/ arylsulfatase	Water	0	10.84%	<LOQ	8.95%	0.53 ± 0.25	13.16%	0.96 ± 0.48	4.59%	101.71 ± 98.03
	Control urine	0	0.80%	<LOQ	0.21%	<LOQ	6.54%	2.19 ± 0.07	1.09%	89.56 ± 54.28
	Control urine	1	1.00%	0.72 ± 0.65	0.18%	6.00 ± 5.93	5.37%	4.71 ± 0.69	0.47%	68.53 ± 9.40
	Control urine	2	1.17%	<LOQ	0.16%	<LOQ	5.61%	4.33 ± 0.55	0.78%	65.11 ± 31.70
	Control urine	4	1.14%	2.16 ± 2.09	0.19%	18.60 ± 18.53	5.76%	5.57 ± 0.05	0.39%	110.25 ± 1.24
	Control urine	16.2	0.67%	7.74 ± 0.83	0.24%	49.58 ± 9.79	4.91%	9.55 ± 0.53	0.31%	193.26 ± 2.52

*Average of 2 replicates

DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

Table 16. Results from liquid-liquid extraction with centrifugal vacuum evaporation.

			d_2-DIM as internal standard		4-methoxy indole as internal standard	
Enzyme treatment	**Sample***	**DIM added (pmol)**	**% recovery**	**DIM (pmol/mL urine or water ± SE)**	**% recovery**	**DIM (pmol/mL urine or water ± SE)**
(-) β-glucuronidase/ arylsulfatase	Control urine	0	33.63%	0.34 ± 0.27	1.01%	67.31 ± 44.79
(+) β-glucuronidase/ arylsulfatase	Control urine	0	11.60%	3.74 ± 0.08	1.01%	166.92 ± 8.47

*Average of 2 replicates
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

Table 17. Results from Experiment 1 accuracy and precision analyses.

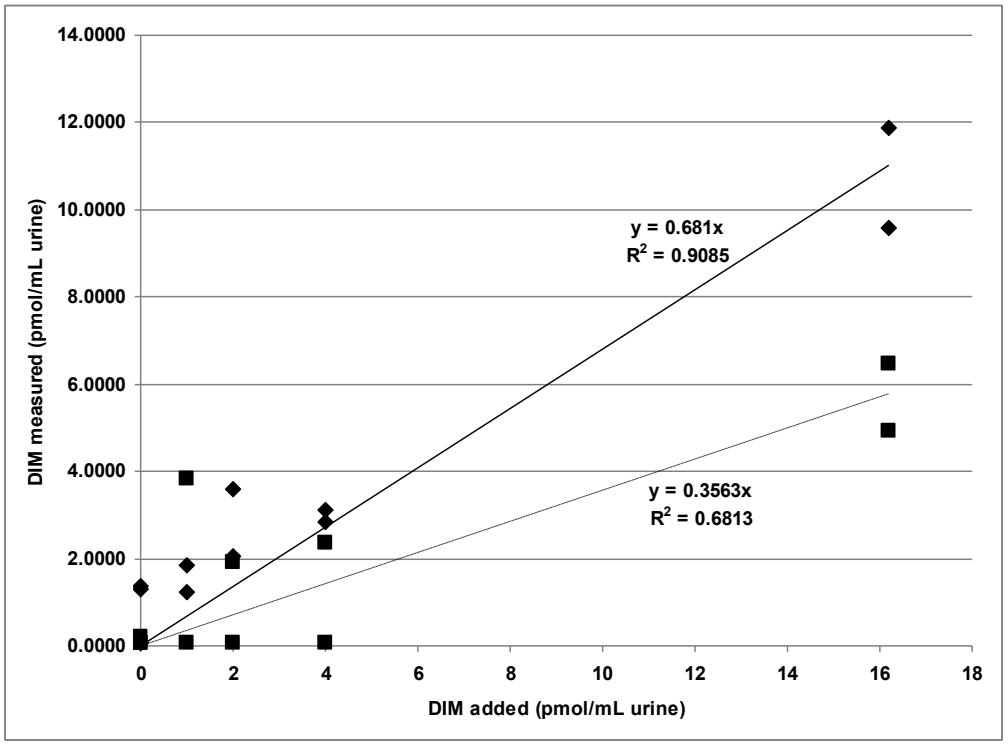
			Solid-phase extraction and centrifugal vacuum evaporation			Liquid-liquid extraction and N_2 evaporation		
Enzyme treatment	**Sample***	**DIM added (pmol)**	**% recovery *d_2*-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**	**% recovery *d_2*-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**
(-) β-glucuronidase/ arylsulfatase	Control urine	2	2.58%	1.15 ± 0.61	117.80%	4.79%	1.03 ± 0.26	56.84%
(+) β-glucuronidase/ arylsulfatase	Control urine	2	0.58%	1.06 ± 0.99	208.85 %	5.48%	2.46 ± 1.10	17.80%

*Average of 5 replicates
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

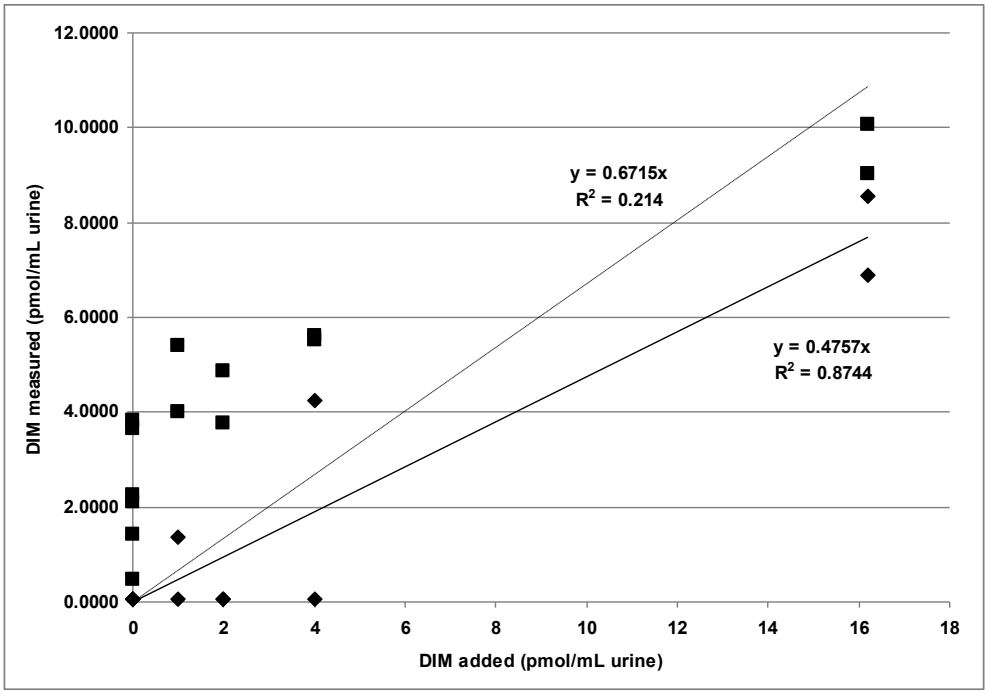
Table 18. Summary of DIM recovery by preparation technique, evaporation method, and enzyme treatment computed via ^{14}C recovery.

Sample preparation technique	**Solid-phase extraction**	**Liquid-liquid extraction**	
Evaporation method	**Centrifugal vacuum evaporation**	**N_2 evaporation**	**Centrifugal vacuum evaporation**
(-) β-glucuronidase/ arylsulfatase[a]	--	61%	69%
(+) β-glucuronidase/ arylsulfatase[b]	21%	21%	23%

[a]Average of 3 replicates
[b]Represents 1 sample, 2 replicates excluded due to contamination



a.



b.

Figure 22. Calibration curves from Experiment 1.
(a) Without β-glucuronidase/arylsulfatase; (b) with β-glucuronidase/arylsulfatase.
◆ Solid-phase extraction; ■ *t*-butyl methyl ether extraction.
——Trendline for solid-phase extraction; – — –trendline for *t*-butyl methyl ether extraction.

Experiment 2: Validation of liquid-liquid extraction technique with physiological concentrations of DIM

Given the results of the ^{14}C recovery experiment, this calibration experiment did not utilize deconjugation enzyme treatment with β-glucuronidase/arylsulfatase. The results presented in Table 19 and Figure 23 demonstrate the improved accuracy (R^2 = 0.999) and reliability (average % coefficient of variation (%CV) = 1.85%) of this technique over the ranges of spiked DIM concentrations (0—40.5 pmol/mL). These results are further borne out by the accuracy and precision analyses conducted with 5.1 pmol DIM (Table 20); the measured DIM concentration of 5.06 ± 0.09 pmol/mL did not differ from 5.1 pmol/mL, and %CV was 3.5% for the four duplicates.

The results of the technique with or without β-glucuronidase/arylsulfatase treatment in the urine of three individuals consuming cruciferous vegetables are shown in Table 21. Although the overall recovery of d_2-DIM is improved in the absence of β-glucuronidase/arylsulfatase (65.7% vs. 18.2% with β-glucuronidase/arylsulfatase), the overall DIM concentrations were increased with the addition of the deconjugation enzyme treatment. When urines were treated with β-glucuronidase/arylsulfatase, the average urinary DIM following cruciferous vegetable intake was 29.25 ± 12.68 compared to 1.25 ± 0.26 pmol/mL without any enzyme treatment, which was borderline statistically significant (p = 0.06), likely due to the small sample size (n=3) and wide variability. This difference suggests that DIM is indeed excreted as a glucuronide and/or sulfonate, and that deconjugation enzyme treatment yields higher levels of urinary DIM than no treatment.

Table 19. Summary of Experiment 2 validating DIM quantification in urine spiked with 0—40.5 pmol DIM

Sample*	**DIM added (pmol)**	**% recovery d_2-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**
Water	0	71.9%	0.18	n/a
Control urine	0	8.1%	0.08 ± 0.01	0.01%
Control urine	5.1	52.9%	5.37 ± 0.05	1.41%
Control urine	10.1	48.3%	9.77 ± 0.17	2.50%
Control urine	20.2	53.8%	19.93 ± 0.37	2.60%
Control urine	40.5	51.4%	40.68 ± 0.79	2.75%

*Average of 2 replicates, except water
Samples were not treated with any enzymes.
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

Table 20. Results from Experiment 2 accuracy and precision analyses.

Sample*	**DIM added (pmol)**	**% recovery d_2-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**
Control urine	5.1	47.91%	5.06 ± 0.09	3.50%

*Average of 4 duplicates
Samples were not treated with any enzymes.

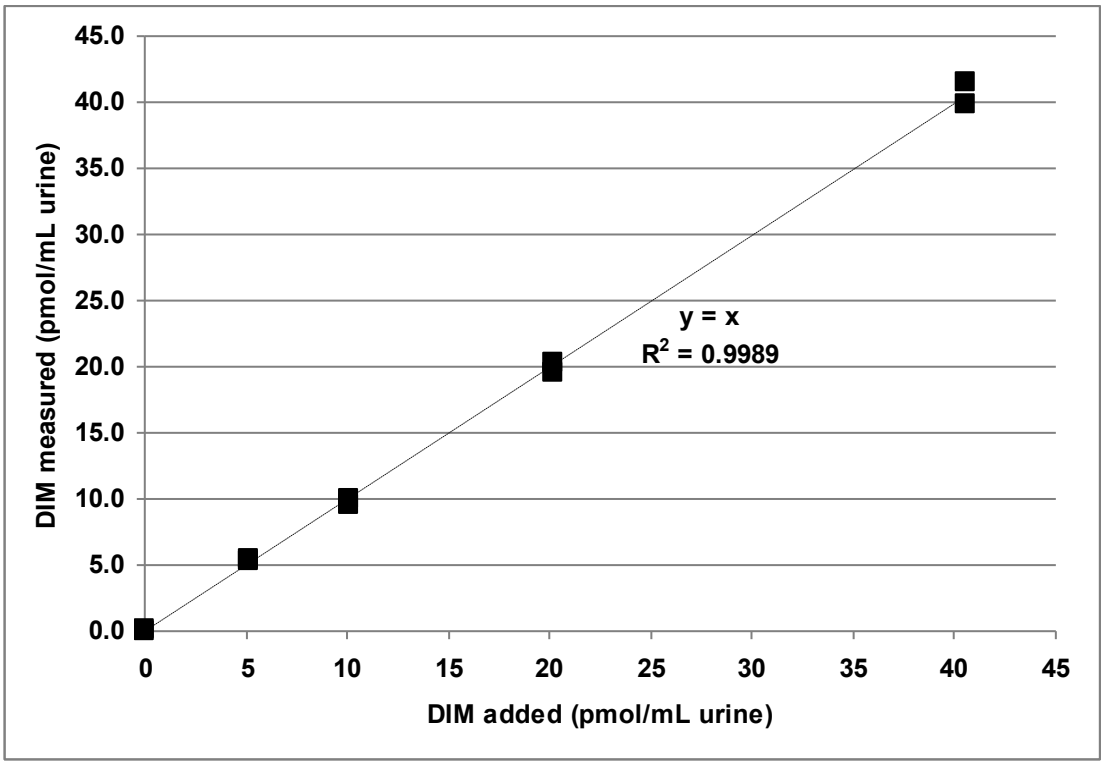


Figure 23. Calibration curve from Experiment 2 *t*-butyl methyl ether extraction without β-glucuronidase/arylsulfatase.
■ *t*-butyl methyl ether extraction.
– — –Trendline for *t*-butyl methyl ether extraction.

Table 21. Summary of urinary DIM concentrations after consuming cruciferous vegetables with or without β-glucuronidase/arylsulfatase treatment.

Enzyme treatment	**(-) β-glucuronidase/arylsulfatase**			**(+) β-glucuronidase/arylsulfatase**		
Subject*	**% recovery d_2-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**	**% recovery d_2-DIM**	**DIM (pmol/mL, mean ± SE)**	**%CV**
A	56.4%	1.30 ± 0.02	2.37%	3.9%	66.14 ± 15.36	32.85%
B	68.7%	0.53 ± 0.24	64.90%	23.9%	2.64 ± 0.18	9.49%
C	72.1%	1.91 ± 0.06	4.25%	26.8%	18.98 ± 1.68	12.56

*Average of 2 replicates
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)
$p = 0.06$ for urinary DIM in cruciferous vegetable versus baseline

Experiment 3: Assessment of inter-day reproducibility and reduced internal standard concentration

The results of quantification of DIM via β-glucuronidase/arylsulfatase treatment, *t*-butyl methyl ether extraction, and centrifugal vacuum evaporation in ten individuals abstaining from or consuming cruciferous vegetables are shown in Table 22. Intake of cruciferous vegetables significantly increased urinary DIM concentrations ($p < 0.05$), as expected. However, in five subjects whose samples were analyzed on three separate days, the variability of urinary DIM measured was quite high, particularly in the baseline urine samples (average %CV 129.70%). The use of supported liquid-liquid extraction by ChemElut cartridges reduced the variability of the baseline analyses somewhat to an average %CV of 75.40% but substantially increased the variability of the cruciferous vegetable urinary DIM concentrations to a %CV of 52.40% (Table 23). Because the goal of these experiments was to validate the urinary DIM technique as a biomarker of cruciferous vegetable intake rather than avoidance, this increase in variability was deemed unacceptable, and supported liquid-liquid extraction was not included in further experiments.

Revisiting Table 22, the addition of 6000 U rather than 2000 U β-glucuronidase/arylsulfatase did not triple the yield as might be expected but rather increased urinary DIM more than 30-fold. This result in addition to detectable levels of DIM in water blanks from each of the three days of the experiment (Table 24) led us to question if contamination had been introduced into the samples by the β-glucuronidase/arylsulfatase preparation. Indeed, the treatment of water with increasing

Table 22. Summary of Experiment 3 results.

	Baseline Urinary DIM (pmol/mL urine)					Cruciferous Vegetable Urinary DIM (pmol/mL urine)*				
	Assay Day					Assay Day				
Subject	1	2	3	Mean ± SE	%CV	1	2	Mean ± SE	%CV	6000 U β-glucuronidase/arylsulfatase
D	2.81	--	--	--	--	7.18	--	--	--	--
E	0.15	--	--	--	--	16.92	--	--	--	--
F	0.29	--	--	--	--	11.73	--	--	--	--
G	0.42	--	--	--	--	13.15	--	--	--	--
H	0.14	--	--	--	--	4.55	--	--	--	--
I	0.35	2.45	20.79	7.86 ± 6.49	143.02%	5.93	8.03	6.98 ± 1.05	21.28%	365.41
J	0.57	0.37	7.19	2.71 ± 2.24	143.16%	2.98	6.42	4.70 ± 1.72	51.68%	245.78
A	0.20	0.45	6.99	2.55 ± 2.22	151.16%	15.92	17.15	16.53 ± 0.62	5.26%	246.36
L	5.95	17.51	37.21	20.22 ± 9.13	78.17%	17.35	16.15	16.75 ± 0.60	5.04%	371.26
M	0.73	3.32	21.52	8.52 ± 6.54	132.98%	6.58	6.90	6.74 ± 0.16	3.31%	248.01

*All subjects consumed 50 g Brussels sprouts for 1 d, except H, who consumed 50 g cabbage.
$p = 0.01$ for urinary DIM in cruciferous vegetable versus baseline urine

Table 23. Results for ChemElut comparison.

	Baseline Urinary DIM (pmol/mL urine)					Cruciferous Vegetable Urinary DIM (pmol/mL urine)*				
	Assay Day				MTBE	Assay Day				MTBE
Subject	1	2	Mean ± SE	%CV	extraction result	1	2	Mean ± SE	%CV	extraction result
D	11.98	1.69	6.84 ± 5.15	106%	2.81	23.04	10.22	16.63 ± 6.41	55%	7.18
E	0.87	1.70	2.83 ± 0.42	46%	0.15	27.14	11.29	19.22 ± 7.92	58%	16.92
G	1.97	3.53	2.75 ± 0.78	40%	0.42	15.22	19.84	17.53 ± 2.31	19%	13.15
I	2.12	5.55	3.83 ± 1.71	63%	7.86 ± 6.49	10.44	12.96	11.70 ± 1.26	15%	6.98 ± 1.05
M	9.16	0.69	4.92 ± 4.23	122%	8.52 ± 6.54	32.18	3.31	17.74 ± 14.43	115%	6.74 ± 0.16

*All subjects consumed 50 g Brussels sprouts on each of 2 d.

Table 24. Summary of daily water blanks prepared via liquid-liquid extraction from Experiment 3.

	DIM (pmol/mL)				
	Assay Day				
	1	2	3	Mean ± SE	%CV
Water	0.54	1.77	13.19	5.17 ± 4.03	135%

Table 25. DIM concentrations in water blanks treated with increasing quantities of *H. pomatia* β-glucuronidase/arylsulfatase

Sample	β-glucuronidase/arylsulfatase (U)	% recovery d_2-DIM	DIM (pmol/mL)
Water	1000	100%	2.80
Water	2000	86%	5.66
Water	4000	84%	10.51
Water	6000	74%	16.64

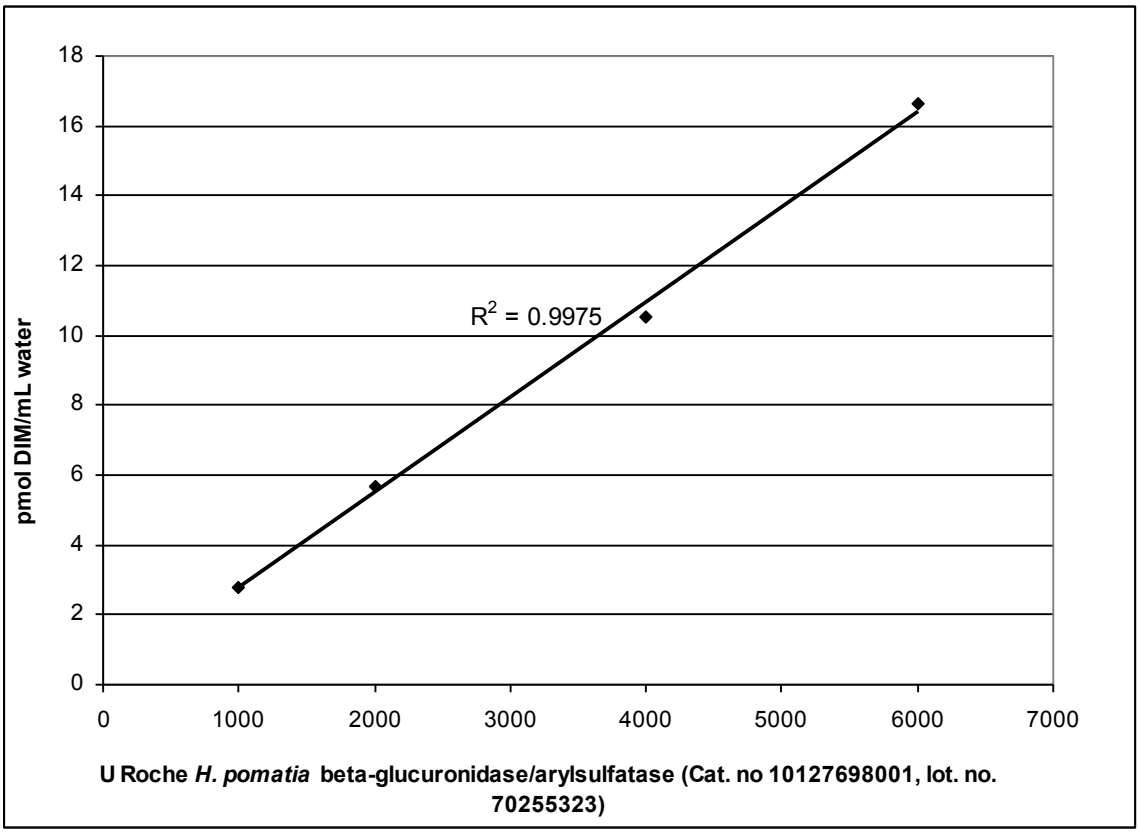


Figure 24. Change in DIM concentration with increasing amounts of *H. pomatia* β-glucuronidase $p = 0.0013$ for linear regression model

quantities of β-glucuronidase/arylsulfatase yielded a significant, positive, and linear association with the quantity of DIM detected (Table 25 and Figure 24).

Experiment 4: Evaluate contamination of H. pomatia *β-glucuronidase/arylsulfatase with DIM*

The concentrations of DIM detected in two separate lots of Roche *H. pomatia* β-glucuronidase/arylsulfatase and β-glucuronidase expressed in *E. coli* (Sigma) are reported in Table 26, and Table 27 summarizes the DIM concentrations detected in various preparation of β-glucuronidase with or without the addition of d_2-DIM. Roche β-glucuronidase/arylsulfatase from *H. pomatia* yielded detectable DIM concentrations, which ranged from 4.41 ± 0.03 to 7.76 ± 2.00 pmol/mL water treated when quantified using d_2-DIM. In contrast, β-glucuronidase expressed in *E. coli* (Sigma) did not elicit quantifiable DIM concentrations, and DIM peaks were only evident in the chromatograms of water treated with d_2-DIM, indicating that the internal standard is the predominant source of DIM in water treated with *E. coli* β-glucuronidase. The percentage of DIM from added d_2-DIM was 0.64%, and this value was used to correct all calculations of total DIM in the following experiments.

Table 26. Concentration of DIM detected in multiple preparations of β-glucuronidase.

β-glucuronidase preparation (2000U)	**DIM (pmol/mL, mean ± SE)**
None[a]	<LOQ
Roche *H. pomatia* lot #70255323[b]	4.41 ± 0.03
Roche *H. pomatia* lot #70331220[c]	6.21 ± 1.21
Sigma *E. coli* G8295[c]	<LOQ

[a]Average of 5 replicates
[b]Average of 4 replicates
[c]Average of 4 replicates
LOQ = limit of quantitation (132 fmol)

Table 27. Summary of Experiment 4 results.

Sample*	β-glucuronidase preparation (2000U)	d_2-DIM added (pmol)	% recovery d_2-DIM	DIM (mean area counts ± SE)	% DIM peak area relative to d_2-DIM peak area ± β-glucuronidase DIM peak area	DIM (pmol/mL water, mean ± SE)
Water	Sigma *E. coli* G8295	0	n/a	nd	n/a	nd
Water	Sigma *E. coli* G8295	10	77.92%	2,199,037 ± 347,979	1.38%	<LOQ
Water	Roche *H. pomatia* lot #70331220	0	n/a	111,614,442 ± 22,110,455	n/a	n/a
Water	Roche *H. pomatia* lot #70331220	10	74.31%	111,356,190 ± 12,144,120	114.89%	7.76 ± 2.00
Water	None	10	100.37%	1,279,627 ± 278,020	0.64%	<LOQ
Water	None	5	94.91%	620,873 ± 133,089	0.64%	<LOQ

*Average of 2 replicates
nd = not detected
LOQ = limit of quantitation (132 fmol)

Experiment 5: Compare β-glucuronidase from H. pomatia *and* E. coli *to no enzyme treatment for DIM quantification and reliability*

Subsequent to concluding that the previous Roche β-glucuronidase/arylsulfatase preparation was contaminated with DIM, urine samples were evaluated with a semi-purified *H. pomatia* β-glucuronidase/arylsulfatase (Sigma H5 G1512), the *E. coli* β-glucuronidase shown to be free of DIM in Experiment 4, or no enzyme to compare the absolute values of DIM quantified in an effort to determine if enzyme treatment was necessary. As shown in Table 28, treatment with either preparation of β-glucuronidase did increase the concentrations of urinary DIM detected, particularly following cruciferous vegetable consumption; however, no individual treatment reached statistical significance from the others. Due to low volumes of urine and H5 β-glucuronidase/arylsulfatase, only the control urine samples were evaluated with this preparation, which did not demonstrate detectable levels of DIM in the baseline urine but increased the quantification of urinary DIM nearly three-fold following cruciferous vegetable consumption.

However, the testing of water blanks with H5 β-glucuronidase/arylsulfatase substantially decreased the recovery of d_2-DIM to 1.68%, resulting in a very high concentration of DIM detected in 1 mL of treated water (48.47 ± 17.02 pmol, Table 29). Conversely, the *E. coli* β-glucuronidase resulted in a high recovery of d_2-DIM (68.04%) with no detectable quantities of DIM in the water samples treated. The improved d_2-DIM recovery in the *E. coli* versus H5 preparation of the enzyme compelled us to choose the *E. coli* preparation of β-glucuronidase for the final urinary DIM quantification technique.

Table 28. Summary of Experiment 5 results comparing yield by enzyme preparation.

		Baseline Urinary DIM (pmol/mL urine)			Cruciferous Vegetable Urinary DIM (pmol/mL urine)		
Subject	**β-glucuronidase preparation (2000U)**	**% recovery d_2-DIM**	**Mean ± SE**	**%CV**	**% recovery d_2-DIM**	**Mean ± SE**	**%CV**
Control[1,*]	None	41.08%	3.28 ± 1.51	113%	37.50%	2.60 ± 0.06	6%
Control[1,*]	Sigma *E. coli* G8295	33.63%	3.20 ± 1.41	108%	24.57%	4.17 ± 0.15	9%
Control[2,*]	Sigma H5 purified *H. pomatia* G1512	25.20%	<LOQ	n/a	21.37%	11.72 ± 0.03	0.5%
D[3]	None	28.52%	0.12 ± 0.04	64%	32.25%	2.26 ± 0.04	2%
D[3]	Sigma *E. coli* G8295	9.47%	6.03 ± 0.19	5%	14.27%	3.19 ± 0.04	2%
E[3]	None	67.41%	<LOQ	n/a	44.88%	4.44 ± 0.41	13%
E[3]	Sigma *E. coli* G8295	30.78%	0.84 ± 0.07	11%	13.46%	11.37 ± 1.80	22%

[1]Average of 6 replicates
[2]Average of 4 replicates
[3]Average of 3 replicates at baseline, 2 replicates following 50 g Brussels sprouts consumption
*Cruciferous vegetable treatment consisted of 200 g Brussels sprouts consumption
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

Table 29. DIM concentrations in water treated with 2000 U β-glucuronidase from *E. coli* or purified from *H. pomatia*.

β-glucuronidase preparation (2000U)*	**% recovery d_2-DIM**	**DIM (pmol/mL water, mean ± SE)**
Sigma *E. coli* G8295	68.04%	<LOQ
Sigma H5 purified *H. pomatia* G1512	1.68%	48.47 ± 17.02

*Average of 2 replicates

Experiment 6: Validate final technique for urinary DIM quantification

This experiment was conducted only with the control urine specimens collected following cruciferous vegetable abstinence and consumption. Two-day inter-assay variability was still relatively high at baseline, with a %CV of 46%, but this value in the urine sample following cruciferous vegetable exposure was reduced to 16% (Table 30). The technique also showed that urinary DIM was higher following cruciferous consumption than abstinence (2.51 ± 0.12 versus 0.89 ± 0.13 pmol, respectively).

The final technique was calibrated in control baseline urine spiked with increasing proportions of urine following cruciferous vegetable exposure (Table 31). Although the results remained somewhat variable, with an average %CV of 9.6%, Figure 25 shows that this technique was indeed valid in quantifying a positive linear association between the amount of cruciferous vegetable exposure and urinary DIM detected ($R^2 = 0.999$). This experiment also validated the technique in relatively low concentrations of DIM, as might be observed in individuals consuming a varied diet with a moderate amount of cruciferous vegetables.

Lastly, the final method was validated in control baseline urine spiked with 1.25—30.35 pmol DIM to evaluate its use with a higher level of exposure to I3C. As shown in Table 32, these results were less variable than in the previous calibration shown in Table 31, with an average %CV of 3 %. Figure 26 confirms that the technique is valid in a range of urinary DIM concentrations ($R^2 = 0.999$).

Table 30. Summary of Experiment 6 results.

	Baseline Urinary DIM (pmol/mL urine) *				Cruciferous Vegetable Urinary DIM (pmol/mL urine) *		
	Assay Day				Assay Day		
	1	2	Inter-day Mean		1	2	Inter-day Mean
% recovery d_2-DIM	11.88%	12.44%	12.14%		22.78 %	21.88%	22.37%
Intra-day Mean ± SE	1.16 ± 0.19	0.62 ± 0.06	0.89 ± 0.13		2.78 ± 0.18	2.24 ± 0.03	2.51 ± 0.12
%CV	37%	21%	46%		14%	3%	16%

*Average of 5 replicates per day

Table 31. Summary of Experiment 6 calibration with control and cruciferous vegetable urine samples.

		Urinary DIM (pmol/mL urine)	
% Cruciferous vegetable urine*	**% recovery d_2-DIM**	**Mean ± SE**	**%CV**
20	15.87%	0.72 ± 0.06	0%
40	13.17%	1.27 ± 0.19	22%
60	13.65%	1.79 ± 0.17	14%
80	12.63%	2.40 ± 0.20	12%

*Average of 2 replicates, cruciferous vegetable treatment consisted of 200 g Brussels sprouts consumption

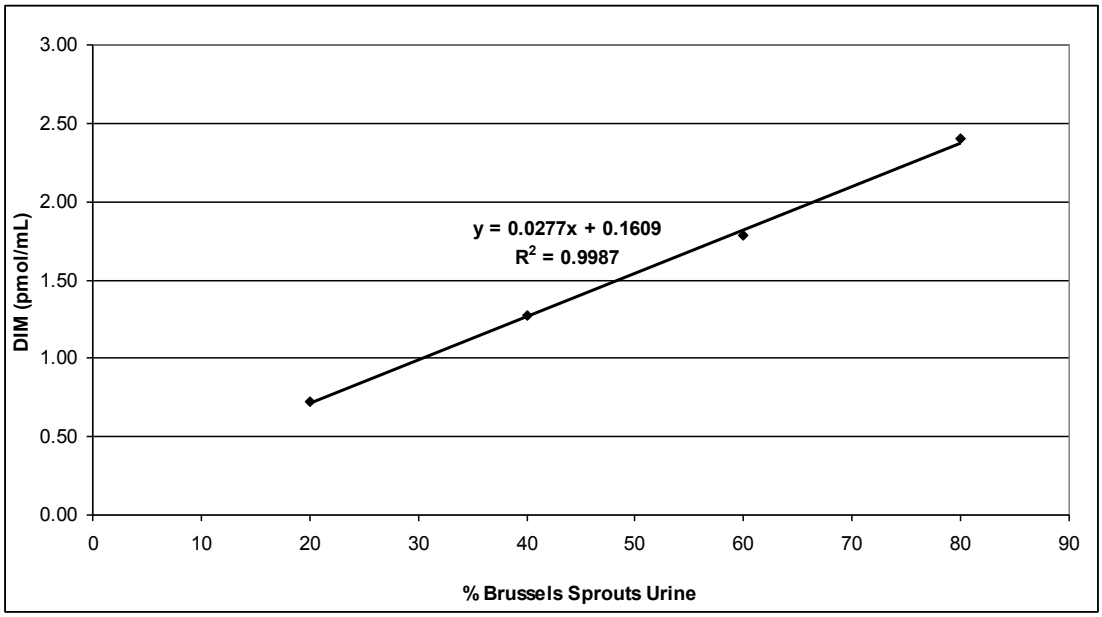


Figure 25. Calibration of urinary DIM method with control urine samples.

Table 32. Summary of Experiment 6 validating DIM quantification in urine spiked with 1.25—30.35 pmol DIM.

DIM added (pmol)*	% recovery d_2-DIM	DIM (pmol/mL, mean ± SE)	%CV
1.25	17.35%	1.40 ± 0.11	11%
2.55	14.20%	2.18 ± 0.04	3%
5.05	18.53%	4.40 ± 0.11	4%
10.1	17.66%	8.97 ± 0.12	2%
20.25	17.10%	18.06 ± 0.11	1%
30.35	11.90%	28.43 ± 0.05	0.2%

*Each sample represents 2 duplicates
DIM concentrations <LOQ (132 fmol) were computed as 50% LOQ (0.07 pmol/mL)

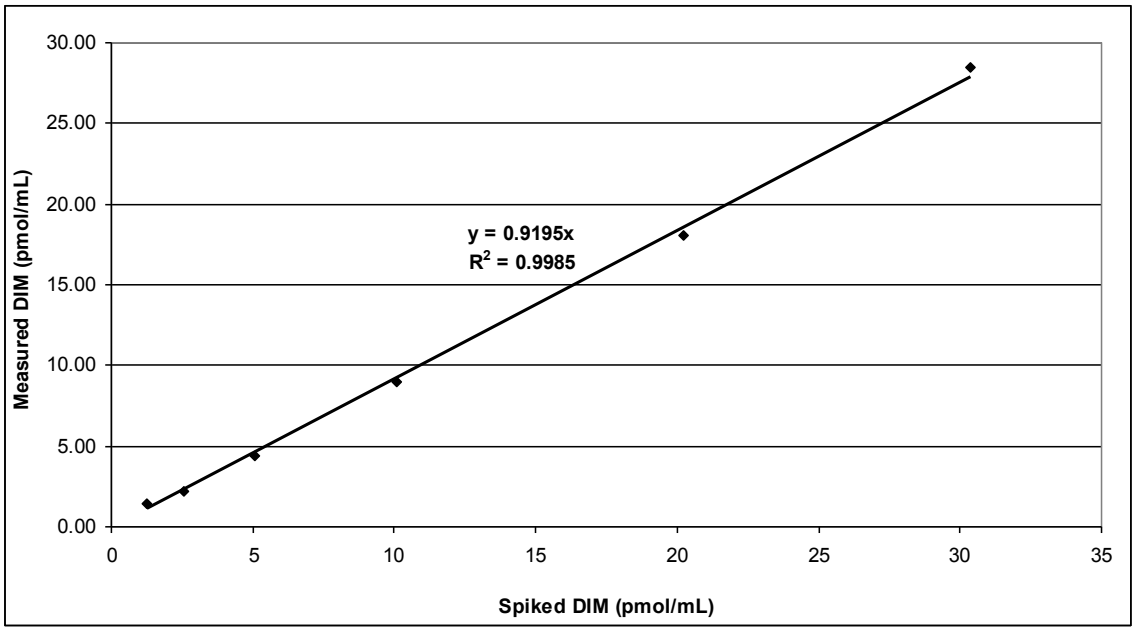


Figure 26. Calibration of urinary DIM method with control urine samples spiked with 1.25—30.35 pmol DIM.

Discussion

The series of experiments described above to validate an LC/MS method to quantify urinary DIM as a biomarker of I3C exposure yielded the final experimental work-up outlined in Figure 27. Several procedures were rigorously tested, and the final technique is highly sensitive, accurate, and reliable. It is a notable improvement to previously published methods because low levels of DIM resulting from cruciferous vegetable consumption rather than DIM or I3C supplementation were detected in the control urine samples. The technique was also accurate and reliable in a range of added DIM concentrations, and the use of deuterated DIM as an internal standard further

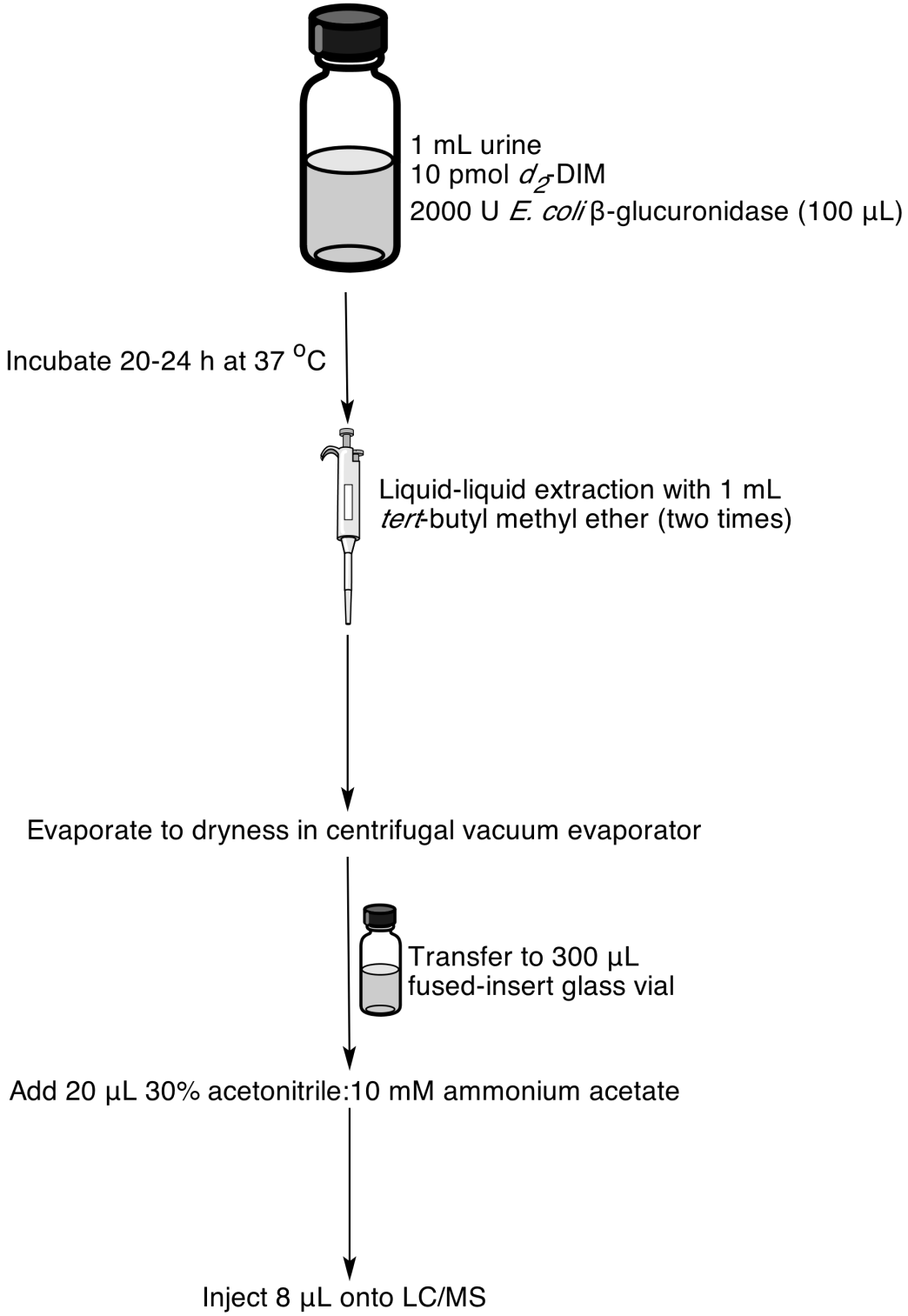


Figure 27. Final urinary DIM sample preparation technique.

increased accuracy by enabling more precise estimations of DIM recovery. Additionally, the use of a recombinant β-glucuronidase expressed in *E. coli* increased the yield of the urinary DIM assay while also eliminating a substantial source of contamination.

Although the technique demonstrated very low variability in urine samples spiked with known amounts of DIM, there remained somewhat wide variability (>10%) in a control urine sample from an individual consuming Brussels sprouts. The low concentrations of DIM present in the urine of this individual likely contribute to this variability, as subtle differences in peak quality and integration can result in large differences in quantitation. The presence of other compounds in the urine resulting from additional metabolites of glucosinolates present in Brussels sprouts or medications consumed by the individual may also contribute to the variability if compounds of similar molecular weight to DIM are excreted. However, given that the same variability was not observed in the same individual's urine spiked with pure DIM, the most plausible explanation seems to be differences in the excreted forms of DIM versus the pure form used for the spiking experiments. For example, I3C can also dimerize to HI-IM (see Figure 14 above), a compound with the same molecular weight as DIM but possibly different stability characteristics in stored samples[171]. Notably, it has not been shown if HI-IM is excreted in the urine. Nevertheless, additional urinary metabolites of I3C may be responsible for variability in urine samples from individuals consuming low amounts of glucobrassicin.

As mentioned previously, an important development during the process of assay validation was the identification of DIM contamination in *H. pomatia*-sourced β-

glucuronidase/arylsulfatase and an alternative source of β-glucuronidase free from contamination (*E. coli*). A majority of the previous human and animal research has utilized *H. pomatia* β-glucuronidase/arylsulfatase, which leads us to question prior results using these methods. Given the potential of DIM to tightly bind proteins, as evidenced by the even higher concentrations detected in *H. pomatia* β-glucuronidase/arylsulfatase purified by size-exclusion chromatography, it would be difficult if not impossible to predict the effect of a contaminated enzyme across multiple heterogeneous urine samples. This precludes the use of corrective equations and requires the re-analysis of all samples with an enzyme free from contamination. The issue of contamination from enzyme sources may not have resulted in appreciable errors historically, but with the increasing use of highly sensitive instruments such as LC/MS, it is imperative to limit contamination as much as possible. Enzyme sources without exposure to the analytes of interest and the use of water blanks with and without internal standards are strongly recommended for accurate research in nutrition and cancer employing highly sensitive biomarkers.

The final method offers an accurate and reliable technique to quantify a range of urinary DIM concentrations resulting from cruciferous vegetables. As will be shown in the next chapter, the technique is sufficient to discern individuals consuming low versus high glucobrassicin-containing vegetables. This innovation is key to observational studies that address large numbers of individuals consuming diets without any experimental control or intervention. An additional benefit of this technique is the use of urine specimens, which are among the least invasive and most easily stored biospecimens.

However, there remain several limitations to the technique described in this chapter. As previously mentioned, the technique is relatively variable at low concentrations of urinary DIM. In addition, the rapid clearance of DIM, which peaks at two hours following a dose of I3C[171], may require multiple urine collections from each individual to accurately assess I3C exposure. Finally in contrast to *H. pomatia* β-glucuronidase/arylsulfatase, *E. coli* β-glucuronidase does not possess any sulfatase activity; the use of sulfatase expressed in *A. aerogenes* may deconjugate an increased concentration of DIM in each sample, but the increased expense associated with multiple engineered enzymes may limit their use in large epidemiological studies.

In conclusion, the series of experiments described above resulted in a valid, reliable, and highly sensitive technique to quantify I3C exposure in individuals consuming vegetables containing glucobrassicin. The method is an improvement to previously published techniques because it does not introduce contamination and can quantify ranges of I3C found in the diet rather than in pharmacological doses. A further improvement to the technique would be its validation in longitudinal I3C exposure. However, the preparation of urine samples with *E. coli* β-glucuronidase and liquid-liquid extraction with *t*-butyl methyl ether followed by quantitation via LC/MS-MS remains a valid technique to quantify urinary DIM as a surrogate marker for dietary glucobrassicin consumption and will be used in current and upcoming controlled feeding trials of cancer chemoprevention by dietary bioactives.

Chapter V: Urinary 3,3'-diindolylmethane: a biomarker of glucobrassicin exposure and indole-3-carbinol uptake in humans

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Introduction

Brassica vegetables from the genus *Brassica* such as broccoli, cabbage, and Brussels sprouts, are unique in that they contain high levels of glucosinolates, widely thought to mediate the anti-cancer effect of these foods[154, 159]. When the plant cells are damaged such as by chewing or chopping, inert glucosinolates are hydrolyzed by plant myrosinase into indoles (indolyl glucosinolates) and isothiocyanates (isothiocyanate glucosinolates)[154, 182, 201]. Only two indolyl glucosinolates are found in abundance in Brassica vegetable crops – glucobrassicin (indolylmethyl) and neoglucobrassicin (methoxy-3-indolylmethyl)[182]. The relative concentration of glucobrassicin is species- and cultivar-dependent and varies according to growth conditions[159]. However, glucobrassicin is the predominant glucosinolate among more than 100 known glucosinolates in many commonly consumed Brassica vegetables[188, 202, 203].

Glucobrassicin is relevant to human health because when plant cells are chewed, chopped, or otherwise damaged, it is converted by endogenous myrosinase into indole-3-carbinol (I3C). In the acidic environment of the stomach, I3C undergoes acid condensation to oligomers, the predominant form and most well characterized of which is 3,3'-diindolylmethane (DIM)[204]. Both I3C and DIM have demonstrated remarkable pleiotropic anti-cancer properties *in vitro* and *in vivo*[199, 205]. Our group has shown that A/J mice treated with the potent tobacco-smoke carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[*a*]pyrene develop significantly fewer lung tumors after treatment with I3C or DIM as compared to those treated with the carcinogens alone[206, 207]. In a placebo-controlled clinical trial, I3C given for 12 weeks resulted in

complete regression of cervical dysplasia in approximately half of the women studied[190]. Other studies have demonstrated both I3C and DIM modulate estrogen metabolism in animals and humans to favor the formation of the anti-proliferative estrogen 2-hydroxyestrone (2-OHE_1) over the pro-proliferative estrogen 16α-hydroxyestrone (16-OHE_1)[191, 193, 194, 208-214]. Indeed, a higher 2-OHE_1:16-OHE_1 ratio is associated with a decreased risk of estrogen-sensitive tumors such as breast cancer[215]. Conversely, prolonged exposure to high doses of I3C can result in dose- and timing-dependent promotion of carcinogen-induced hepatocarcinogenesis in rainbow trout and rat models[216, 217]. A study in rats found that I3C at doses up to 20 mg/kg daily by gavage for 28 days or thirteen weeks was not toxic but higher doses induced liver toxicity despite withdrawal of I3C[218]. However, the highest non-toxic dose in this study (20 mg/kg body weight/day) exceeds the maximum tolerated dose of I3C in humans of 400 mg daily[192].

Epidemiological evidence links a high intake of Brassica vegetables to a reduction in the risk of lung, stomach, bladder, prostate, and other cancers[156, 219-227]; however, the association has not been consistently replicated, highlighting the necessity of developing biomarkers to quantify the uptake of the phytonutrients within such vegetables[160, 228-232]. Several biomarkers of isothiocyanate exposure have been evaluated in humans[182, 188, 233-244]. However, no published study has successfully correlated consumption of an intact vegetable to glucobrassicin and I3C metabolites in humans. We conducted a randomized, crossover trial with the objective of testing whether urinary DIM is a biomarker for the uptake of I3C from Brassica vegetables

containing divergent glucobrassicin concentrations. The hypothesis being tested is that glucobrassicin exposure and subsequent I3C uptake is directly related to DIM excreted in the urine. To measure urinary DIM, we developed a novel and powerful liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC/ESI-MS/MS-SRM) method and compared urinary DIM levels after consumption of a Brassica vegetable with relatively high glucobrassicin concentration ('Jade Cross' Brussels sprouts) to urinary DIM levels after consumption of a Brassica vegetable with relatively low glucobrassicin concentration ('Blue Dynasty' cabbage).

Materials and Methods

Study population

Healthy, non-vegetarian, non-smoking adults ages 18 to 65 were recruited. Former smokers were required to be abstinent from tobacco for at least one year. Non-smoking status was confirmed by exhaled carbon monoxide levels. Subjects with medical conditions requiring the active care of a physician, pregnant or breastfeeding women, vegetarians, and those taking warfarin were excluded. Subjects completed questionnaires on medical history, medication/supplement use, demographics, and smoking history (former smokers). The protocol and consent form were approved by the Institutional Review Board at the University of Minnesota. All subjects provided informed consent.

Study design

This was a randomized, two-period crossover trial. The study schema is shown in Figure 28. All 25 subjects completed study procedures concurrently. Subjects were asked to abstain from Brassica vegetables and glucosinolate-containing foods starting seven days prior to consumption of the first study vegetable (washout period 1) to the end of the study. Compliance was ascertained from self-reported food diaries and DIM level in baseline spot urine samples collected at the end of each washout period, immediately prior to consumption of the study vegetable. Subjects were randomized to consume either 50 g of raw 'Jade Cross' Brussels sprouts or 50 g of raw 'Blue Dynasty' cabbage in a single sitting at the study center once every 24 h for three consecutive days (Phase 1). All urine was collected for each 24 h period after study vegetable feeding. After a five-day washout period (washout period 2), subjects consumed 50 g of Brussels sprouts or cabbage (whichever they did not consume before) at the study center once every 24 h for three consecutive days (Phase 2). Urine was collected in the same manner as before. All urine was measured and aliquoted the day of collection, and stored at -20°C until analysis. Fasting was not required throughout the study period.

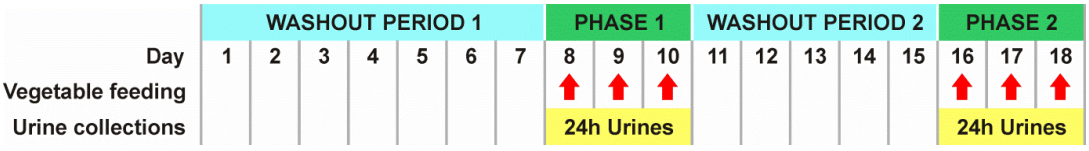

Figure 28. Crossover feeding study design.
⬆Vegetable feeding
Brussels sprouts or cabbage were fed once daily for three consecutive days on days 8-10 and 16-18. All urine was collected for each 24 h period after vegetable feeding. The sequence of vegetable feeding was randomly assigned to each subject at the time of enrollment. Subjects remained abstinent from cruciferous vegetables, other than those fed, starting with the first washout period through study completion on day 19.

Cultivation of 'Jade Cross' Brussels sprouts and 'Blue Dynasty' cabbage

'Jade Cross' Brussels sprouts and 'Blue Dynasty' cabbage (Jordan Seeds, Woodbury, MN) were selected due to their divergent glucobrassicin concentrations (unpublished data). The vegetables were seeded in 48-cell trays (53 × 27 cm) containing moist soilless seeding media (Sunshine SB-300 Universal; Sun-Gro Horticulture, Bellevue, WA) for 28 days. Following an eight-day hardening off period, seedlings were planted 46 cm apart in Waukegan silt loam (deep, well-drained glacial soil, 0–1% slope; pH = 7.4, 48 ppm phosphorous, 148 ppm potassium, 3.7% organic matter) in Rosemount, MN (44.7°N). Soil was fertilized with 168 kg ha^{-1} nitrogen (urea) and treated with 0.84 kg ha^{-1} trifluralin herbicide (incorporated) prior to planting. Plants were watered as needed with drip irrigation throughout the season. Cabbage and Brussels sprouts stalks were harvested after a total of 65 and 98 days of growth, respectively. Two days after stalk harvest, U.S. No. 1 & 2 grade Brussels sprouts were removed from the stalks[245]. Approximately 125 g from four different cabbage heads and approximately 200 g from three different Brussels sprouts were taken for glucobrassicin analysis as described below. Produce was stored in ventilated plastic bags at approximately 5ºC.

Preparation of 'Jade Cross' Brussels sprouts and 'Blue Dynasty' cabbage

Vegetables were delivered to the study center 45 days and 12 days after harvest, respectively, on day one of Phase 1 of the study. One-half of four cabbage heads was cored, and ~125 g from each half were taken for glucobrassicin analysis. The remainder of the cabbage halves was chopped into bite-sized pieces (~25 cm^2) to make 50 g salads,

and 120 g samples (n=3) of these salads were analyzed for glucobrassicin concentration. Brussels sprouts were washed, the discolored leaves and the stem were removed, and the sprouts were halved to make 50 g salads. Approximately 100 g (n=3) of these salads were taken for glucobrassicin analysis. Both cabbage and Brussels sprout salads were prepared fresh each day prior to administration throughout the study. Vegetable preparation and glucobrassicin quantification of the salads only was done in an identical manner at the start of Phase 2 of the study.

Analysis of glucobrassicin concentration in the vegetables

Vegetable samples were prepared for glucobrassicin analysis on the day of collection. Sample preparation consisted of boiling the samples in water to deactivate myrosinase, blending and homogenizing samples (2 min, 12,000 RPM; Polytron PT 1300D; Brinkmann Instruments, Westbury, NY), centrifuging to remove debris, extracting desulpho-glucobrassicin using strong anion-exchange (SAX) solid-phase extraction (SPE), and filtering the extract through a 0.2 μm PTFE syringe filter (pre-wetted with methanol) before sample storage at -30ºC. Samples were stored at -10ºC after blending until homogenization. Further details of the methods are described elsewhere[246, 247]. HPLC analysis was carried out based on an existing protocol with minor modifications[246]. The injection volume was 50 μL. Analyses were done using an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a solvent degasser and diode array detector. The solvent gradient was as follows (A = H_2O, B = acetonitrile): 0 to 2 min, 5 to 15% B; 2 to 20 min, 15 to 47% B; 20 to 23 min,

47 to 100% B; 23 to 30 min, 100% B; 30 to 33 min, 100 to 5% B. Peaks were verified using known retention times and absorbance spectra, and integrated using Chemstation software (Agilent Technologies). Desulpho-GB was quantified using a desulphosinigrin standard curve and a response factor of 0.29[248].

Chemicals

All chemicals were HPLC, LC-MS, or Optima grade. DIM was purchased from LKT Labs (Saint Paul, MN). Indole, ammonium acetate, *t*-butyl methyl ether, and β-glucuronidase preparations from *Escherichia coli* (G8295) were purchased from Sigma-Aldrich (St. Louis, MO). d_2-Formaldehyde (isotopic purity 98%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Synthesis of d_2*-DIM*

The synthesis of d_2-DIM was carried out essentially as previously described[200]. Briefly, d_2-formaldehyde (20% w/w aqueous solution; 1.5 mL) was added to indole (1.17 mg, 0.01 mol) suspended in a mixture of glacial acetic acid (0.6 g, 0.01 mol) in 2.5 mL of D_2O. The mixture was vigorously stirred under nitrogen at 90°C for 5 h, cooled, and purified using the HPLC system described below. The retention time of d_2-DIM was 41 min (0.6 g, 50%, and isotopic purity greater than 99%). ^{1}H NMR (d_6-DMSO) showed absence of methylene protons, δ 10.7 (2H, 1NH, 1′NH), 7.5 (d, J = 15.6 Hz, 2H, 4H, 4′H), 7.25 (d, J = 16.2 Hz, 2H, 7H, 7′H), 7.18 (d, J = 3.6 Hz, 1H, 2H, 2′H), 7.05 (dd, J = 1.8, 14.4 Hz, 2H, 5H, 5′H), 6.95 (dd, J = 1.2, 14.4 Hz, 2H, 6H, 6′H). The MS

(positive ion ESI) showed the following: *m/z* (relative intensity) 249 [M+1] (25); 131[M-indole-C-d_2].

HPLC system

The HPLC system consisted of a Waters model 680 gradient controller, two Waters 510 pumps, a Waters 440 absorbance detector (254 nm) (Waters, Milford, MA), a Hewlett Packard 1100 series autosampler (Palo Alto, CA), and a Luna C18 reverse-phase column 250 x 4.6 mm, 5 μm (Phenomenex, Torrance, CA) with a linear gradient from 10% CH_3OH in H_2O to 100% CH_3OH in 40 min and held for an additional 10 min at a flow rate of 0.7 mL/min.

Preparation of urine samples for DIM analysis

Urine samples were prepared using a published technique with modifications[196, 249]. Two thousand units of *E. coli* β-glucuronidase solution in 0.1 mL of phosphate buffered saline (0.26%, w/v) and 10 pmol d_2-DIM internal standard were added to 1 mL of each urine sample. Following a 20 h incubation at 37°C, each sample was extracted two times with an equal volume of *t*-butyl methyl ether. The extracts were evaporated to dryness using a centrifugal vacuum evaporator (Savant SpeedVac, Thermo Fisher Scientific, Waltham, MA) and reconstituted to 20 μL with acetonitrile/10 mM ammonium acetate (30/70, v/v). Positive and negative control urine samples and water blanks were included with each assay.

Quantitation of urinary DIM by LC-ESI-MS/MS-SRM

The analyses were carried out by LC-ESI-MS/MS-SRM on a TSQ Quantum Discovery Max instrument (Thermo Fisher Scientific, Waltham, MA) in the positive ion mode with N_2 as the nebulizing and drying gas. MS parameters were set as follows: spray voltage, 3.2 kV; sheath gas 25; capillary temperature, 250°C; collision energy, 17 V; scan width, 0.05 amu; Q2 gas pressure 1.0 mTorr; source CID 9 V; tube lens offset, 104 V; Q1 0.2 amu and Q3 0.7 FWHM. MS data were acquired and processed by Xcalibur software version 1.4 (Thermo Electron, Waltham, MA). Eight μL of the sample were injected from an autosampler using an Agilent 1100 capillary LC system (Agilent Technologies) equipped with a 5μm, 150 x 0.5 mm Zorbax SB-C18 column (Agilent Technologies). The flow rate was15 μL/min for the first 3 min then 10 μL/min with a gradient from 60% methanol in 15 mM NH_4OAc to 100% methanol in 8 min and held for additional 29 min. The mass transitions monitored were *m/z* 247.12→130.07 for DIM (typical retention time of 16.6 min) and *m/z* 249.12→132.07 for d_2-DIM (typical retention time of 16.5 min). Quantitation was done by comparing the MS peak area ratio of DIM to that of d_2-DIM using a calibration curve prepared before each analysis. The calibration curve was prepared by plotting the MS peak area ratio of DIM to d_2-DIM against their concentration ratios using standard mixes containing constant levels of d_2-DIM and varying levels of DIM. The assay limit of quantitation (LOQ) was 132 fmol (32.5 pg). Results below the assay LOQ were computed as 50% LOQ (0.07 pmol/mL).

Accuracy and precision of urinary DIM quantification

Accuracy and precision were determined using two 24 h urine collections from an individual who abstained from Brassica vegetables for seven days (negative control urine) and then consumed 200 g of commercial Brussels sprouts (positive control urine). Five replicates of 1 mL of positive control urine treated with 2000 units of *E. coli* β-glucuronidase and 5 pmol d_2-DIM were analyzed on two different days to determine the accuracy, reliability, and reproducibility of the assay. To validate the method over a range of DIM concentrations, 1.25, 2.55, 5.05, 10.1, 20.25, or 30.35 pmol of pure DIM was added to 1 mL of negative control urine and analyzed in duplicate.

Urine creatinine measurements

Urine creatinine in each urine collection was measured at the University of Minnesota Medical Center, Fairview Diagnostic Laboratatory, a CLIA-certified lab.

Statistical analysis

Log-transformed means were compared using t-tests. A linear regression model was used to analyze the effect of body mass index (BMI), sex, age, and race/ethnicity on urinary DIM. The coefficient of variation (%CV) was calculated as standard deviation/mean x 100. A 5% significance level was used for all tests. Analysis was done using SAS 9.2 (Cary, NC).

Results

Subject characteristics

Ten males and 15 females (total n=25) ranging in age from 22 to 63 years (mean 35.8 ± 11.8 years) completed the study. There were 20 Caucasians, two African Americans, and three Asians. The average BMI was 26.7 ± 5.0. Twenty subjects (80%) were never smokers. All subjects consumed the vegetable salads as scheduled. No adverse events were observed. No subjects were taking proton pump inhibitors, H_2-antagonists, other antacids, or antibiotics. Compliance was excellent. Six of the 150 twenty four-hour urine collections (4%) from five different subjects were missing one partial urine void per collection. There were no major devations abstaining from Brassica vegetables during the study period as ascertained from self-reported food diaries and baseline urinary DIM levels.

Glucobrassicin concentration

The glucobrassicin concentration in the vegetables analyzed at three timepoints during the study is shown in Table 33. The mean glucobrassicin concentration in the cabbage was approximately four-fold lower than in the Brussels sprouts at all sampling timepoints, and remained consistent from the time of harvest to the time of administration.

Table 33. Glucobrassicin coneentration (± SEM) of harvested vegetables and salads.

	µmol glucobrassicin/100 gfw			
	Harvest	**Phase 1**	**Phase 2**	**Mean**
Cabbage	47.4 ± 13.3	44.2 ± 1.4	45.3 ± 2.0	45.8 ± 5.0*
Brussels sprouts	165.3 ± 2.2	194.8 ± 6.4	191.6 ± 5.5	183.9 ± 6.4

Halved cabbages (n=4) were sampled at harvest. Brussels sprouts were sampled on the day they were separated from stalks, 2 days after harvest (n=3). Brussels sprout and cabbage salads were sampled on the first day of each phase (n=4 and 3, respectively). The mean glucobrassicin concentration was significantly higher in Brussels sprouts than in cabbage (*p <0.001). Abbreviations: gfw = gram food weight.

Accuracy and precision of urinary DIM quantification

Accuracy data are shown in Figure 29. Accuracy averaged 93% (range 86-112%). The intra-day precision averaged 8.5%, and the inter-day variability averaged 16% (n=5 positive control urine samples for each). Duplicates were evaluated at each concentration of negative control urine to which known amounts of DIM were added, and precision averaged 3%. The correlation between the amount of DIM added to the urine and the amount measured was excellent (R^2 = 0.999).

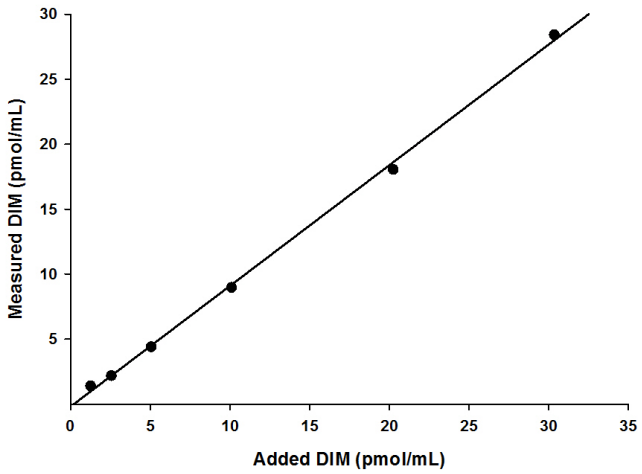

Figure 29. Measured versus added DIM in negative control urine samples to which known amounts of DIM were added. R^2=0.999. Slope = 0.92.

Analysis of urinary DIM levels

Typical LC-ESI-MS/MS-SRM chromatograms obtained at baseline and after consumption of Brussels sprouts are illustrated in Figure 30. Urinary DIM was undetectable in 38 of the 50 baseline urine samples; nine subjects produced a peak at the retention time of DIM. Baseline urinary DIM levels were not significantly different after each washout period when analyzed according to the order of vegetable feeding ($p = 0.89$ for Period 1, $p = 0.85$ for Period 2). No period or carryover effects were observed ($p = 0.21$ and 0.27, respectively).

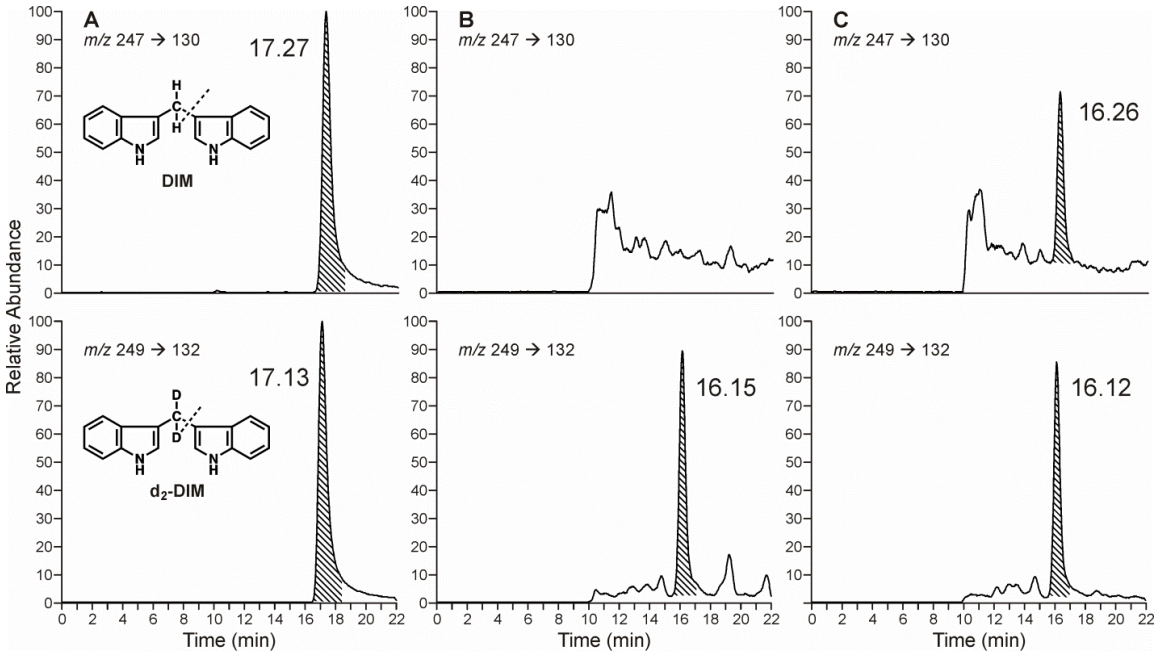

Figure 30. Representative LC/MS traces.
Representative LC-ESI-MS/MS-SRM chromatograms obtained upon analysis of A, standard DIM (top) and internal standard d_2-DIM (bottom); B, a urine sample from a subject before eating Brussels sprouts (top) and internal standard d_2-DIM (bottom), and C, a urine sample from a subject after eating Brussels sprouts (top) and internal standard d_2-DIM (bottom).

Mean 24 h urinary DIM level in each of the 25 subjects is summarized in Table 34, and was consistently higher after consumption of either cabbage or Brussels sprouts compared to baseline (p <0.001 for both). Within each subject, Brussels sprouts consumption consistently led to higher mean urinary DIM than cabbage consumption. The mean difference was 8.64 ± 1.65 pmol/mg creatinine (range 1.40 – 42.53 pmol/mg creatinine). As shown in Figure 31, among the entire group, overall mean urinary DIM after consumption of Brussels sprouts was significantly higher than the overall mean urinary DIM after consumption of cabbage (geometric mean 7.07 ± 1.14 pmol/mg creatinine vs. 0.29 ± 1.22 pmol/mg creatinine, p <0.0001). The excreted urinary DIM averaged 0.03 ± 0.01% (Brussels sprouts) and 0.01 ± 0.00% (cabbage) of the glucobrassicin administered.

Wide inter-individual variability was observed within each type of vegetable feeding. Mean urinary DIM ranged from 1.40 to 44.00 pmol/mg creatinine after Brussels sprouts feeding and 0 to 1.47 pmol/mg creatinine after cabbage feeding. Urinary DIM was undetectable in four subjects after cabbage feeding. Mean urinary DIM after Brussels sprouts in these four subjects was 5.76, 5.22, 8.89, and 5.76 pmol/mg creatinine, respectively.

The potential effect of subject characteristics on urinary DIM after Brussels sprouts feeding was analyzed using a linear model that included BMI, ethnicity, and age. Mean urinary DIM did not correlate with these variables (p = 0.23 for overall model).

Table 34. Mean 24 h urinary DIM among each of the 25 subjects after consumption of Brussels sprouts or cabbage once daily for 3 consecutive days.

Subject #	Mean 24 h Urinary DIM (pmol/mg creatinine)		Feeding order
	B	C	
1	9.18	0.60	C
2	9.83	0.44	B
3	44.0	1.47	C
4	5.33	1.03	B
5	5.40	0.24	B
6	5.84	0.42	B
7	13.1	0.56	B
8	11.7	0.62	B
9	5.17	0.28	B
10	5.22	0.00	C
11	21.8	0.85	C
12	4.88	0.05	C
13	7.02	0.12	C
14	15.6	0.41	B
15	5.17	0.17	C
16	3.08	0.20	B
17	8.89	0.00	C
18	5.22	0.51	B
19	1.40	0.00	B
20	7.58	0.12	C
21	6.12	0.48	B
22	10.2	0.56	C
23	5.76	0.00	C
24	4.03	0.14	C
25	4.66	0.92	B
Overall	9.05 ± 1.70	0.41 ± 0.07	

Each value represents the mean of three 24 h urine collections. Feeding order refers to which vegetable – Brussels sprouts or cabbage – the subject was randomized to eat during Phase I of the study. Overall mean values are reported ± standard error. Abbreviations: C, cabbage; B, Brussels sprouts

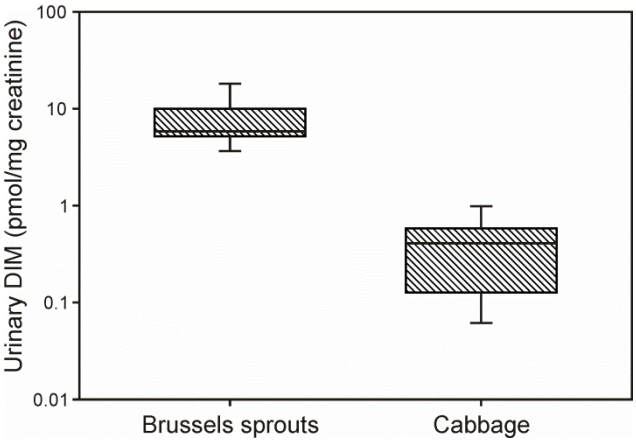

Figure 31. Overall mean urinary DIM among the 25 subjects after consumption of Brussels sprouts or cabbage, after feeding once daily for 3 consecutive days (p <0.0001).

Discussion

This study is the first to successfully quantify urinary DIM, a biomarker of I3C uptake, after feeding Brassica vegetables, and the first to show that higher glucobrassicin exposure from food consistently results in higher I3C uptake and higher urinary DIM levels as hypothesized. This proof-of-principle is essential to establish urinary DIM as a non-invasive, reproducible measure of I3C uptake from Brassica vegetables that can be applied in prospective epidemiological and chemoprevention studies. Our highly sensitive LC/ESI-MS/MS-SRM technique makes it possible to quantify the relatively small quantities of DIM in urine that result from food consumption, an obstacle not encountered with I3C or DIM supplements, which are dosed at levels significantly higher than can be achieved with food[193].

Characterizing I3C uptake using urinary DIM is advantageous. The glucosinolate concentration in the same cultivar of Brassica vegetable can vary based on factors such as growth conditions and preparation technique, making random estimations of

glucosinolate exposure impractical[202, 239, 240]. Measuring glucosinolate uptake based on the type and quantity of Brassica vegetable consumed is also unreliable, and is a potential factor in the inconsistent correlation between high Brassica vegetable consumption and decreased cancer risk observed in some epidemiologic studies. Furthermore, measurement of I3C itself is problematic since I3C readily undergoes oligomerization in the acidic conditions of the stomach and is undetectable in blood within one hour after I3C administration to both mice and humans[171, 192].

Our method of measuring urinary DIM represents a notable improvement to previously published assays[191-193, 196, 214, 249]. The use of d_2-DIM as internal standard results in more precise identification of DIM as it is structurally more similar to DIM compared with previously used internal standards such as 4-methoxyindole or 4,4'-dichloro-DIM. Additionally, we used recombinant *E. coli* β-glucuronidase, which ameliorated the confounding from *H. pomatia*-derived β-glucuronidase observed during our assay development and in assays used by others[250-252].

Previous studies have attempted to characterize I3C uptake after Brassica vegetable feeding. In one study, healthy volunteers were fed blanched broccoli, selenium-fortified blanched broccoli, or placebo once daily for four weeks[196]. Blood and urine were collected prior to the intervention and the day following the last broccoli feeding. The glucobrassicin concentrations in the diet were 111 and 107 μg/g fresh weight of broccoli and selenium-fertilized broccoli, respectively. Neither DIM nor I3C was detectable in the urine or blood. The authors hypothesized that this was due to factors such as the ascorbic acid content in the vegetables favoring the formation of ascorbigen,

or the ~24 h duration between the end of the intervention and sample collection. Other studies have analyzed DIM in plasma or urine after administration of I3C or DIM supplements. After dosing I3C at 200 mg daily for four weeks (n=5), mean urinary DIM was 12.1 ± 2.5 (SD) μg/mg creatinine (49,125 pmol/mg creatinine, approximately 5,000 times greater than in our subjects who ate Brussels sprouts), which was not significantly different from mean urinary DIM after 400 mg daily for four weeks[191]. Absorption-enhanced DIM (BioResponse-DIM®, BioResponse LLC, Boulder, CO) dosed at 108 mg per day for 30 days resulted in a significant increase in mean urinary DIM (collected the day following the final DIM dose) to 101.3 ± 36.1 ng/mg creatinine[193]. Finally, after dosing patients with thyroid proliferative disease intending to undergo thyroidectomy with absorption-enhanced DIM at 300 mg daily for fourteen days, the mean increase in urinary DIM was 383.5 ng/mg creatinine[214]. The current study resulted in substantially lower urinary DIM levels, consistent with the micro-dosing achievable from food.

The proportion of glucobrassicin consumed that was excreted in the urine as DIM was low. A major factor is likely a low rate of conversion of I3C to DIM in the stomach due to the complex heterogeneity of gastric contents after eating. Additionally, the major route of I3C or DIM excretion in humans is not known. Several studies have examined the tissue distribution and metabolism of I3C based on administration of radiolabeled I3C in animal models, but did not directly quantify I3C or DIM[170, 253, 254]. In F344 rats, after administering [^{3}H] I3C in the diet for seven days, the feces was the predominant route of excretion of the recovered radiolabel (75%); only 23% was excreted in the

urine[170]. In mice, after administration of 250 mg/kg of I3C once by gavage, the concentration of I3C and DIM was highest in liver, then kidney[171]. Overall, the data suggest that I3C and DIM may not be readily absorbed by the gut or are excreted primarily by biliary excretion, as suggested in rainbow trout[254].

The widely variable inter-individual difference in urinary DIM excretion was expected, and is consistent with other studies after administration of I3C, DIM, or cruciferous vegetables[192, 239, 242, 255, 256]. We did not require subjects to fast before or after vegetable feeding sessions, which could favor heterodimerization of I3C to higher-order oligomers owing to differences in gastric pH or contribute to variability in I3C/DIM absorption. We also did not require a uniform method of chewing, which might affect myrosinase release, although the extent of minimal vegetable preparation to reach an edible sample was kept consistent for this reason. Taken together, our findings and those of others suggest that inter-individual variations in urinary DIM excretion are probably explained by uncharacterized factors that may have an implication on the relative benefit an individual might derive from Brassica vegetable consumption. Furthermore, the relative chemopreventive effect is likely to be heterogeneous based on polymorphisms in genes encoding Phase I and II biotransformation enzymes (i.e. *CYP* and *GST*), which are modulated by glucosinolates[154, 257-263]. Further studies are required to examine these hypotheses.

Other important questions remain before urinary DIM can be widely used as a biomarker of I3C uptake from Brassica vegetables. First, the pharmacokinetics of I3C and DIM after Brassica vegetable consumption must be delineated. Second, like any

biomarker with a relatively short half-life, use of urinary DIM as a biomarker of an individual's Brassica vegetable intake may need to be assessed longitudinally. Finally, it is not clear whether the benefit of glucobrassicin exposure reaches a plateau nor do we know what a biologically relevant dose in humans may be. Further study into these questions is underway.

In summary, urinary DIM is an objective measure of assessing I3C uptake from Brassica vegetables containing divergent glucobrassicin concentrations. The development of a highly sensitive LC/ESI-MS/MS-SRM method and the results of our study provide a strong foundation to further validate urinary DIM for use in larger populations and in chemopreventive interventions using Brassica vegetables.

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Part III: Dietary fish, fat, and antioxidant consumption and the risk of gastric cancer in the Singapore Chinese Health Study

Introduction

Gastric cancer is among the deadliest malignancies worldwide. Although infection of the stomach with *Helicobacter pylori* (*H. pylori*) is an established risk factor for gastric cancer, environmental exposures including diet have been shown to modulate the risk of gastric cancer with or without *H. pylori* infection. Diets rich in anti-inflammatory compounds, such as n-3, may reduce the risk of gastric cancer by reducing inflammation or triggering cancer cell death; however, these data are somewhat limited *in vitro* and in animal models, and results have been contradictory in human observational studies. Additional longitudinal research examining dietary fat, fish, and antioxidant intakes and gastric cancer risk in an established prospective cohort would yield valuable information regarding the effects of fish, fat, and antioxidant intakes on gastric cancer risk.

Chapter VI: Literature Review

Prevalence of and risk factors for gastric cancer

Gastric cancer is the third leading cause of cancer death in men and fifth leading cause of cancer death in women worldwide[264]. In the United States, the age-standardized rates for all gastric cancers are 7.0 and 3.2 per 100,000 men and women, respectively[265]. While rates of non-cardia gastric cancer have been steadily declining in developed countries, the rates of cancer affecting the gastric cardia have been increasing[266]. Recently, it was reported that the rates of adenocarcinoma of the esophagogastric junction, including cancer of the gastric cardia, are increasing the most rapidly of any solid tumors in Europe and North America[267].

The rates of gastric cancer are especially high in Asian countries such as Japan due in part to traditional diets rich in salt and fermented foods[268, 269]. Although the incidence rates of gastric cancer in Singapore do not reach the levels of Japan (46.8 per 100,000 males, 18.2 per 100,000 females) and Korea (62.2 per 100,000 males, 24.6 per 100,000 females), the age-standardized rates for men and women are much higher than in the United States, at 21.5 and 10.8 per 100,000, respectively[265, 270]. A similar trend of decreasing non-cardia and increasing cardia gastric cancers has also been observed in Singapore, where it was recently reported that 16% of all gastric cancers affect the cardia, double the proportion reported from 1993-1997[267].

Infection of the stomach with *Helicobacter pylori* (*H. pylori*) is a risk factor for gastric cancer, and the International Agency for Research on Cancer classified infection

as a group 1 human carcinogen in 1994[271]. Many large epidemiological studies have concluded that *H. pylori* infection significantly increases the risk for non-cardia gastric cancer[269, 272, 273]. However, the role of *H. pylori* infection in cardia gastric cancer is less clear, possibly due to historically low numbers of cases and the preferential colonization of the gastric antrum by *H. pylori*[271, 274].

Although *H. pylori* infection is a strong risk factor for non-cardia gastric cancer, dietary intake modulates the risk of both non-cardia and cardia gastric cancer. Studies have shown that high salt intake interacts synergistically with *H. pylori* infection to increase the risk of non-cardia gastric cancer, while a few studies demonstrated a significant protective effect of vitamin C or fresh fruit for all gastric cancers irrespective of *H. pylori* status[273, 275]. Additionally, consumption of allium vegetables, such as garlic and onions, has been shown to reduce the risk of gastric cancer[266]. One possible explanation for the latter effect is the sulfur provided by allium vegetables contributing to the production of the endogenous antioxidant glutathione (GSH, see Chapter 1).

Because gastric cancer etiology is linked strongly to chronic inflammation, it is conceivable that the anti-inflammatory effects of dietary n-3 may reduce the risk of such cancers[271]. Additionally, gastric tissue is unique with respect to exposure to lipid peroxidation metabolites that may serve to up-regulate antioxidant and detoxification functions and eliminate cancer cells (see Chapter 1); lipid peroxidation can occur at gastric pH levels[276]. Consequently, the stomach would be exposed to lipid peroxidation metabolites found in food products, formed during the digestion of dietary n-3, and from n-3 present within its cellular membranes.

In an exploration of the association between fish and dietary n-3 consumption and the risk of gastric cancer, a recent meta-analysis by Wu et al.[277] concluded that high fish consumption reduced the risk of gastric cancer by thirteen percent (odds ratio (OR) = 0.87, 95% CI = 0.71 – 1.07) based upon data from 15 case-control and two prospective cohort studies. Because of the limited data from prospective cohort studies, an analysis of the Singapore Chinese Health Study is warranted. In this study of more than 60,000 individuals, detailed dietary information was collected at baseline prior to the diagnosis of any cancer, and participants were followed for an average of twelve years[278-280]. Biospecimens (including blood) were collected from a randomly selected three percent of the subjects at baseline beginning in 1994 and from all consenting subjects in 2000. By 2005, 32,543 subjects had provided biospecimens (28,330 bloods, 4400 buccal cells from blood refusals, and 31,965 urines). This number represents a consent rate of approximately 60% once subjects who had died were excluded. All extracted components from the biospecimens have been stored in -80°C freezers[278]. The collection of biospecimens allows for *H. pylori* testing to control for this important risk factor, which may be present in 43% of Singapore Chinese individuals[278, 281].

Dietary fish, fat, and antioxidant consumption and the risk of gastric cancer

In vitro *and animal model studies*

Few primary *in vitro* and animal model studies have been conducted to explore the relationship between diet and gastric cancer risk, and a majority of the literature on this topic has been derived from epidemiological studies, which will be reviewed in the

next section. A study conducted by Miwa et al.[282] tested the effect of a high-fat (15% of energy) or low-fat (0.5% of energy) diet on male Wistar rats following a partial gastrectomy or sham surgery as control. The rats consuming the high-fat diet following the gastrectomy exhibited a significantly increased incidence of gastric carcinoma (41% of rats fed the high-fat diet versus 15% of rats fed the low-fat diet, $p < 0.05$) and adenoma (38% of rats fed the high-fat diet versus 15% of rats fed the low-fat diet, $p < 0.05$) compared to the rats consuming the low-fat diet. However, the rats that received the sham surgery did not develop any tumors, regardless of diet; the authors attributed this effect to the absence of duodenogastric reflux following the partial gastrectomy that exposed the experimental rats to carcinogenic *N*-nitroso compounds. Additionally, the high amounts of LA present in the soybean oil used for the diets (52.7% by weight) likely promoted inflammation following the surgery.

Cross-sectional and ecological studies

Studies addressing the relationship between dietary fat, fish, and antioxidant intake and the risk of gastric cancer via cross-sectional or ecological designs are summarized in Table 35. Two cross-sectional studies of Swedish fishermen and their wives were conducted by Rylander, Hagmar, and Svensson et al.[283, 284] to examine differences in mortality rates and causes in these individuals compared to the general population of Sweden. The authors found a significantly higher rate of gastric cancer incidence (standardized incidence ratio (SIR) = 1.59, 95% CI 1.03—2.39) and a non-significantly increased rate of gastric cancer mortality among east coast Swedish

Table 35. Summary of cross-sectional and ecological human studies examining dietary fat, fish, and antioxidant intakes and the risk of gastric cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Rylander and Hagmar[284]	Cross-sectional fisherman's wives in Sweden n=8594 n=400 with dietary interviews Expected mortality and cancer incidences calculated for each county based on death and population statistics	Cause-specific mortality ratios Standardized cancer incidence ratios	No difference in overall mortality in fisherman's wives compared to the general population. Cohort members on the east coast had sig. increased breast cancer mortality. There were trends for decreased ischemic heart disease and cancer mortality in the west coast cohort. There were no differences in stomach cancer incidences, but a low number of cases were expected.	East coast fishermen shown to have higher levels of dioxin-like organochlorine compounds than west coast fishermen.
Svensson et al.[283]	Cross-sectional Swedish fishermen n=11,373 Expected mortality and cancer incidences calculated for each county based on death and population statistics	Cause-specific mortality ratios Standardized cancer incidence ratios	East coast fisherman had significantly reduced overall mortality and cardiovascular disease mortality compared to the general population. There was a trend for decreased cancer incidence among east coast fisherman, but there was a non-significant increase in stomach cancer deaths compared to the general population. Stomach cancer incidence was sig. higher among east coast fishermen than the general population (standardized incidence ratio (SIR)=1.59, 95%CI 1.03—2.39). West coast fishermen had significantly reduced overall mortality compared to the general population. There was a significant increase in death from ischemic heart disease. There were no differences in stomach cancer rates compared to the general population. The east coast fishermen had significantly increased incidences of stomach cancer compared to the west coast fishermen (incidence rate ratio (IRR)=2.15, 95%CI 1.32—3.50).	East coast fishermen shown to have higher levels of dioxin-like organochlorine compounds than west coast fishermen. East coast fishermen consume smoked fish twice as often as west coast fishermen. East coast fishermen smoked more than west coast fisherman or the general population.
Johnson et al.[285]	Cross-sectional retrospective cohort of American seafood workers, n=4116	Cause of death via National Death Index, Social Security Administration, state Departments of Vital Records,	Significant excess in deaths due to gastric cancer among the seafood workers (proportional mortality rate (PMR)= 2.2, 95% CI 1.2—3.8), particularly among females (PMR=3.3, 95% CI 1.7—6.3). PMR for occlusion or stenosis of the pre-cerebral or cerebral arteries were significantly lower among the cohort members (PMR=0.5, 95% CI 0.3—0.9).	No dietary (or other environmental exposure) information collected.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
		Departments of Motor Vehicles, personal contact, and internet tracing Standardized US mortality rates from University of Pittsburgh database	Among white females, deaths due to ischemic heart disease were significantly lower among seafood workers compared to the general population (standardized mortality ratio (SMR)=0.8, 95% CI 0.6—1.0).	
Palli et al.[286]	Cross-sectional study of tissue samples from gastric cancer patients n=105	*p53* mutation prevalence Dietary and lifestyle factors from in-person interview	*p53* mutations detected in 31.4% of tissues, more commonly in cardia or fundus tumors and intestinal or unclassified tumors; mutations also more common among women and older subjects. There were no associations between mutations and *H. pylori* infection, smoking, socioeconomic status, education level, and family history of gastric cancer. There were significant associations between increasing consumption of raw vegetables (lettuce, chicory, and carrots) and decreased *p53* mutations (OR=0.2, 95%CI 0.1—0.9) and increasing consumption of nitrites (OR=3.4, 95%CI 1.0—11.4) and increased *p53* mutations. Traditional soups (OR=40.5, 95%CI 5.7—504), nitrite (OR=16.5, 95%CI 1.8—151.2), protein (OR=9.8, 95%CI 1.04—93.8), animal protein (OR=14.3, 95%CI 1.4—143.5), and animal lipids (OR=12.0, 95%CI 1.5—93.9) were associated with an increased risk of non-CpG mutations. Total lipids were borderline associated (OR=5.8, 95%CI 0.9—38.1, $p = 0.066$)	Histological determination of *H. pylori* infection may underestimate proportion affected.
Moore et al.[287]	Ecological study of Western countries n=34 countries	Age- and sex-specific mortality from cancers, diseases of the circulatory system, and cerebrovascular disease from the	There were significant positive correlations between cerebrovascular disease and gastric cancer incidence in both men ($r = 0.7649$, $p < 0.001$) and women ($r = 0.0559$, $p < 0.001$) across countries.	Hypertension considered a risk factor for gastric cardia cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
		World Health Organization Heart disease mortality calculated by subtracting cerebrovascular disease mortality from circulatory disease mortality		
Cai et al.[288]	Ecological study of Fujian Province, China n=55 townships	In-person interview about lifestyle and dietary habits and history of gastric diseases	There was a significant positive correlation between fish sauce consumption and gastric cancer mortality in both men (r = 0.5170, p <0.01) and women (r = 0.4261, p <0.01). The significant positive association persisted after multivariate adjustment (men: p = 0.0001, women: p = 0.0019).	Fish sauce is salted and produced by prolonged fermentation. It has been shown to be carcinogenic.
Ahn et al.[289]	Cross-sectional study of cancerous and noncancerous gastric tissue from gastric cancer patients n=9	Stomach mucosa fatty acid composition	Relative saturated fatty acids (cancer:noncancer = 1.08, p = 0.05) and PUFA were sig. higher (cancer:noncancer = 1.35, p = 0.002) and MUFA sig. lower (cancer:noncancer = 0.72, p = 0.001) in cancerous vs. noncancerous tissues. Arachidonic acid (cancer:noncancer = 1.99, p = 0.0005) and n-6:n-3 sig. higher in cancerous vs. noncancerous tissues (cancer:noncancer = 2.27, p = 0.003, in phospholipids). Total n-3 sig. lower in cancerous vs. noncancerous tissues phospholipids (cancer:noncancer = 0.43, p = 0.04). Higher desaturation indices of n-6 in cancerous vs. noncancerous mucosa.	

fishermen compared to the general population (SIR = 1.37, 95% CI 0.82—2.23)[283]. Compared to the west coast fishermen, those living and working on the east coast had more than twice the rate of gastric cancer incidence (incidence rate ratio (IRR) = 2.15, 95% CI 1.32—3.50). The authors cited evidence that east coast fishermen have higher levels of dioxin-like organochlorine compounds detected compared to west coast fishermen as an explanatory factor; additionally, the east coast fishermen smoked more cigarettes and consumed more smoked fish than the west coast fishermen. When the authors studied the wives of the fishermen, no significant differences in gastric cancer incidence or mortality were observed, although dietary interviews showed that the fishermen's wives consumed approximately twice as much fish as the general population[284].

A similar cross-sectional retrospective cohort study was conducted in American seafood workers by Johnson et al.[285]. When compared to the general population, there was a significant excess in deaths due to gastric cancer among the seafood workers (proportional mortality rate (PMR) = 2.2, 95% CI 1.2—3.8), particularly among females (PMR = 3.3, 95% CI 1.7—6.3). However, it was not known if seafood consumption among these workers differed from the general population. It is noteworthy that the PMR for occlusion or stenosis of the pre-cerebral or cerebral arteries were significantly lower among the seafood workers (PMR = 0.5, 95% CI 0.3—0.9), and among white female seafood workers, deaths due to ischemic heart disease were significantly lower among seafood workers compared to the general population (standardized mortality ratio (SMR) = 0.8, 95% CI 0.6—1.0). These differences may be due in part to the beneficial effects of

seafood consumption on cardiovascular and cerebrovascular diseases, but it is impossible to determine this from the data provided[2, 5].

In an ecological study that did not directly assess nutritional risk factors, Moore et al.[287] reported significant positive correlations between cerebrovascular disease and gastric cancer incidence in both men (correlation coefficient (r) = 0.7649, p <0.001) and women (r = 0.0559, p <0.001) in 34 Western countries. Given the relationship between dietary factors and cardiovascular disease risk, this link (although practically nonexistent among women) may be partially explained by dietary intake and intimates a negative association between fatty fish intake and gastric cancer incidence. Cai et al.[288] conducted an ecological study across 55 townships in the Fujian province of China to explore the correlation between dietary habits and gastric diseases. These authors demonstrated a significant positive correlation between fish sauce consumption and gastric cancer mortality among men (r = 0.5170, p <0.01) and women (r = 0.4261, p <0.01). The fish sauce that is consumed in this region is produced via prolonged fermentation and had been shown previously to be carcinogenic, suggesting that *N*-nitroso compounds present in the fish sauce confer an increased risk of gastric cancer.

Two cross-sectional studies included in Table 35 conducted biochemical analyses of gastric tissue from gastric cancer patients. Palli et al.[286] linked self-reported dietary habits to *H. pylori* infection and *p53* mutations among 105 gastric cancer patients, and concluded that although there were no significant associations between *H. pylori* infection and *p53* mutation prevalence, significantly increased prevalence of *p53* mutations were seen in individuals that consumed higher amounts of traditional soups,

polenta, and nitrites (OR in multivariate model = 3.4, 95% CI 1.0—11.4). More frequent consumption of raw vegetables was associated with a significantly decreased prevalence of *p53* mutations (OR = 0.2, 95% CI 0.1—0.9). In a subgroup analysis of *p53* mutations not occurring in cytosine- and guanine-rich segments of DNA (non-CpG mutations), traditional soups (OR = 40.5, 95% CI 5.7—504), nitrite (OR = 16.5, 95% CI 1.8—151.2), protein (OR = 9.8, 95% CI 1.04—93.8), animal protein (OR = 14.3, 95% CI 1.4—143.5), and animal lipids (OR = 12.0, 95% CI 1.5—93.9) were associated with substantially increased odds of *p53* mutations. There was a borderline positive association between the highest quintile of total lipids intake and non-CpG mutations (OR = 5.8, 95% CI 0.9—38.1, $p = 0.066$). These results suggest that—independently of *H. pylori* infection—dietary factors including high sodium foods, animal fats and protein, and food sources of nitrite may promote gastric cancer via mutations of the tumor suppressor gene *p53*. However, the sub-analysis of non-CpG *p53* mutations was conducted in a small number of subjects (n=14), resulting in very wide confidence intervals that may not apply to larger populations.

In a small study of nine gastric cancer patients, Ahn et al.[289] compared the fatty acid composition of cancerous versus noncancerous gastric mucosa. Compared to the noncancerous tissues, cancerous tissues had significantly higher relative amounts of saturated fatty acids (cancer:noncancer = 1.08, $p = 0.05$) and PUFA (cancer:noncancer = 1.35, $p = 0.002$) and significantly lower relative amounts of monounsaturated fatty acids (MUFA, cancer:noncancer = 0.72, $p = 0.001$) measured from total fatty acids. Specifically, AA was significantly higher (cancer:noncancer = 1.99, $p = 0.0005$) and the

n-6:n-3 ratio was relatively similar in total fatty acids but significantly higher in phospholipids (cancer:noncancer = 2.27, $p = 0.003$) in cancerous versus noncancerous tissues. Total phospholipid n-3 were significantly lower (cancer:noncancer = 0.43, $p = 0.04$). Although this study was conducted with a very small number of subjects, the results suggest an association between gastric mucosa fatty acid composition and gastric cancer progression, with phospholipids low in n-3 relatively enriched in cancerous gastric tissues. Nevertheless, a limitation of all the studies included in Table 35 is the inability to infer causation as all measurements are conducted at one time point only.

Case-control studies

Many case-control studies have explored the associations between dietary fat, antioxidants, and gastric cancer risk, and these studies are summarized in Table 36. A majority of the studies assessed dietary intake and environmental exposures through an in-person interview with a food frequency questionnaire (FFQ) in individuals recently diagnosed with gastric cancer and control individuals without gastric cancer. Compared to cross-sectional and ecological studies, case-control studies offer more information regarding the dietary determinants of cancer risk because they are retrospective in design; however, case-control studies are also subject to several limitations, in particular recall bias, which was defined in Chapter 1. Because of the high mortality rate of gastric cancers, a number of the case-control studies utilized proxy interviews of family members or friends of the cancer patient, likely contributing additional information biases.

Table 36. Summary of case-control human studies examining dietary fat, fish, and antioxidant intakes and the risk of gastric cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Kabat et al.[290]	Hospital-based case-control Distal esophageal/gastric cancer cases, n=173 Non-GI or tobacco-related cancer patient controls, n=4544	In-person food frequency questionnaire Tobacco, alcohol, and supplement use and occupational exposures assessed via interview	Male current (OR=2.3, 95%CI 1.4—3.9) and ex-smokers (OR=1.9, 95%CI 1.2—3.0) at significantly higher odds for gastric cardia cancer than never smokers. Female current smokers at significantly higher odds for both gastric cardia (OR=4.8, 95%CI 1.7—14.0, vs. never smokers) and distal stomach cancer (OR=3.2, 95%CI 1.3—7.7, vs. never smokers). Alcohol consumption was associated with significantly higher odds for gastric cardia cancer among men only (OR=2.3, 95%CI 1.3—4.3 for 4+ oz whisky equivalents/d vs. nondrinkers); the effect was significant for occasional hard liquor only (OR=1.8, 95%CI 1.2—2.7, vs. nondrinkers). In women, daily beer consumption was significantly associated with increased odds for gastric cardia cancer (OR=4.9, 95%CI 1.1—22.8, vs. nondrinkers). There was a significant trend for increased gastric cardia cancer odds with increasing fat intake (OR=2.9, 95%CI 1.5—5.6, highest vs. lowest quartile, *p* for trend <0.001). A similar trend was seen with vitamin A from animal sources (OR=2.4, 95%CI 1.3—4.6, highest vs. lowest quartile, *p* for trend <0.01). Increasing fiber intake was associated with a significant decrease in gastric cardia cancer (OR=3.2, 95%CI 1.5—7.0, lowest vs. highest, quartile, *p* for trend <0.01).	30-item food frequency questionnaire
Ji et al.[291]	Population-based case-control Gastric cancer cases, n=1124 Frequency-matched controls, n=1451	In person dietary and lifestyle interview	Significant trend for increased gastric cancer odds with increasing preserved vegetable intake in women only (OR=1.9, 95%CI 1.3—2.8, highest vs. lowest quartile, *p* for trend = 0.002). Significant inverse trend for poultry intake and gastric cancer in men only (OR=0.7, 95%CI 0.5—0.9, highest vs. lowest, *p* for trend = 0.0005). Significant inverse trends for all vegetables (OR=0.4, 95%CI 0.3—0.5, highest vs. lowest, *p* for trend <0.0001), yellow/green vegetables (OR=0.5, 95%CI 0.4—0.7, highest vs. lowest, *p* for trend = 0.0001), and	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			soybean and products (OR=0.5, 95%CI 0.4—0.7, highest vs. lowest, *p* for trend = 0.0001) in men only. Significant inverse trends for egg (men: OR=0.6, 95%CI 0.4—0.8, highest vs. lowest, *p* for trend = 0.001; women: OR=0.5, 95%CI 0.4—0.8, highest vs. lowest, *p* for trend = 0.003), fresh fruit (men: OR=0.4, 95%CI 0.3—0.6, *p* for trend <0.0001; women: OR=0.5, 95%CI 0.3—0.8, highest vs. lowest, *p* for trend = 0.0006), and plant oil (men: OR=0.7, 95%CI 0.5—0.9, highest vs. lowest, *p* for trend = 0.001; women: OR=0.6, 95%CI 0.4—0.9, highest vs. lowest, *p* for trend = 0.005) intake and gastric cancer in both sexes. Significant inverse association between protein (men: OR=0.7, 95%CI 0.5—0.9, highest vs. lowest, *p* for trend = 0.003; women: OR=0.6, 95%CI 0.4—1.0, highest vs. lowest, *p* for trend = 0.01) and fiber (men: OR=0.6, 95%CI 0.4—0.8, highest vs. lowest, *p* for trend = 0.002; women: OR=0.6, 95%CI 0.3—0.9, highest vs. lowest, *p* for trend = 0.007) intake and gastric cancer risk in both sexes. Significant positive association between carbohydrate (men: OR=1.5, 95%CI 1.1—2.1, highest vs. lowest, p for trend=0.002; women: OR= 1.9, 95%CI 1.3—2.9, highest vs. lowest, *p* for trend = 0.0007) intake and gastric cancer risk in both sexes. Among women, there was a significant inverse association between fat intake and gastric cancer odds (OR=0.6, 95%CI 0.4—0.8, highest vs. lowest, *p* for trend = 0.006). In men, ascorbic acid (OR=0.5, 95%CI 0.3—0.7, highest vs. lowest, *p* for trend <0.0001), carotene (OR=0.4, 95%CI 0.3—0.6, highest vs. lowest, *p* for trend <0.0001), riboflavin (OR=0.4, 95%CI 0.3—0.6, highest vs. lowest, *p* for trend <0.0001), vitamin A (OR=0.7, 95%CI 0.5—1.0, highest vs. lowest, *p* for trend = 0.02), and vitamin E (OR=0.5, 95%CI 0.3—0.7, highest vs. lowest, *p* for trend <0.0001) were significantly associated with reduced gastric cancer odds. In women, significant negative associations were seen only with carotene (OR=0.7, 95%CI 0.5—1.1, highest vs. lowest, *p* for	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			trend = 0.02), riboflavin (OR=0.5, 95%CI 0.3—0.9, highest vs. lowest, *p* for trend = 0.003), and vitamin E (OR=0.5, 95%CI 0.3—0.8, highest vs. lowest, *p* for trend = 0.0002). In both sexes, increasing frequency of salted food consumption (overall: OR=1.7, 95%CI 1.3—2.4, frequently vs. occasionally, *p* for trend = 0.001; distal: OR=1.8, 95%CI 1.3—2.7, frequently vs. occasionally, *p* for trend = 0.002), preference for saltiness (overall: OR=2.3, 95%CI 1.0—4.9, very salty vs. low salty, *p* for trend = 0.0005; distal: OR=1.5, 95%CI 0.6—4.0, very salty vs. low salty, *p* for trend = 0.008), and increasing consumption of fried foods (overall: OR=2.3, 95%CI 1.6—3.2, frequently vs. occasionally, *p* for trend = 0.0001; distal: OR=2.0, 95%CI 1.3—3.0, frequently vs. occasionally, *p* for trend = 0.004) were significantly associated with increased overall and distal gastric cancer odds. Increasing temperature of soup or porridge (overall: OR=1.9, 95%CI 1.5—2.5, burning hot vs. not hot, *p* for trend <0.0001; cardia: OR=2.9, 95%CI 1.7—4.9, burning hot vs. not hot, *p* for trend <0.0001; distal: OR=1.9, 95%CI 1.4—2.5, burning hot vs. not hot, *p* for trend <0.0001), frequency of irregular meals (overall: OR=3.6, 95%CI 2.7—4.9, frequently vs. never, *p* for trend <0.0001; cardia: OR=4.0, 95%CI 2.4—6.5, frequently vs. never, *p* for trend <0.0001; distal: OR=3.7, 95%CI 2.7—5.2, frequently vs. never, *p* for trend <0.0001), speed of eating (overall: OR=2.0, 95%CI 1.8—2.5, fast vs. moderate, *p* for trend <0.0001; cardia: OR= 3.8, 95%CI 2.5—5.7, fast vs. moderate, *p* for trend <0.0001; distal: OR=1.8, 95%CI 1.5—2.2, fast vs. moderate, *p* for trend <0.0001), and propensity for binge eating (overall: OR= 2.5, 95%CI 2.0—3.1, yes vs. no; cardia: OR=3.0, 95%CI 2.0—4.4, yes vs. no; distal: OR=2.4, 95%CI 1.9—3.1, yes vs. no) were significantly associated with increased odds for overall, cardia, and distal gastric cancers. Frequent consumption of raw and fresh foods was significantly associated with reduced odds for overall (OR=0.6, 95%CI 0.5—0.8, frequently vs. occasionally, *p* for trend = 0.0001),	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			cardia (OR=0.5, 95%CI 0.3—1.0, frequently vs. occasionally, *p* for trend = 0.02), and distal (OR=0.6, 95%CI 0.4—0.8, frequently vs. occasionally, *p* for trend = 0.003) gastric cancers.	
Kaaks et al.[292]	Population-based case-control Gastric cancer cases, n=301 Population controls, n=2851	In-person food frequency questionnaire	Overall, total protein (OR=0.64, highest vs. lowest quartile, *p* for trend = 0.024), total vegetable protein (OR=0.53, highest vs. lowest quartile, *p* for trend = 0.001), total polysaccharides (OR=0.63, highest vs. lowest quartile, *p* for trend = 0.002), linoleic acid (OR=0.53, highest vs. lowest quartile, *p* for trend <0.001), dietary fiber (OR=0.31, highest vs. lowest quartile, *p* for trend <0.001), vitamin C (OR=0.43, highest vs. lowest quartile, *p* for trend <0.001), beta-carotene (OR=0.50, highest vs. lowest quartile, *p* for trend <0.001), vitamin B1 (thiamin, OR=0.27, highest vs. lowest quartile, *p* for trend <0.001), vitamin B3 (niacin, OR=0.54, highest vs. lowest quartile, *p* for trend <0.001), and vitamin B6 (OR=0.39, highest vs. lowest quartile, *p* for trend <0.001) were significantly associated with decreased risk for gastric cancer. Total energy (OR=2.19, highest vs. lowest quartile, *p* for trend <0.001), total mono- and disaccharides (OR=1.88, highest vs. lowest quartile, *p* for trend <0.001), retinol (OR=2.26, highest vs. lowest quartile, *p* for trend <0.001), and vitamin B2 (riboflavin, OR=1.58, highest vs. lowest quartile, *p* for trend = 0.004) were associated with significantly increased risk for gastric cancer. Associations for MUFA (OR=0.75, highest vs. lowest quartile, *p* for trend = 0.064) and PUFA (OR=0.69, highest vs. lowest quartile, *p* for trend = 0.065) were borderline significantly inverse.	
Ye et al.[293]	Population-based case-control Gastric cancer cases, n=272 Population controls, n=544	In-person food frequency questionnaire and lifestyle habits interview	Consumption of fish sauce (OR=2.57, 95%CI 1.89—3.50), 0.4+ kg/m vs. <0.4 kg/m, *p* <0.01), moldy foods (OR=2.32, 95%CI 1.73—3.09, yes vs. no, *p* <0.01), irregular dinners (OR=5.47, 95%CI 4.22—7.09, 3+ times/w vs. <3 times/w, *p* <0.01), salted vegetables (OR=1.41, 95%CI 1.09—1.83, 2+ kg/y vs. <2 kg/y, *p* <0.05), salted fermented sea foods (OR=1.57, 95%CI 1.21—2.02, 1.5+ kg/y vs. <1.5 kg/y, *p*	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			<0.01), and family history of cancer (OR=3.27, 95%CI 2.48—4.31, yes vs. no, *p* <0.01) significantly increased the risk of gastric cancer in univariate analysis. Consumption of green tea (OR=1.72, 95%CI 1.26—2.36, ≤0.75 kg/y vs. >0.75 kg/y, *p* <0.01), citrus fruits (OR=1.41, 95%CI 1.03—1.92, ≤2.5 kg/y vs. >2.5 kg/y, *p* <0.05), other fruits (OR=1.31, 95%CI 1.01—1.71, ≤2.5 kg/y vs. >2/5 kg/y, *p* <0.05), and fresh vegetables (OR=1.95, 95%CI 1.41—2.70, ≤25 kg/y vs. >25 kg/y, *p* <0.01) significantly decreased the risk of gastric cancer in univariate analysis. Results for fish sauce (adjusted attributable risk (AAR)= 17.81%, *p* <0.01), irregular dinners (AAR=48.93%, *p* <0.01), salted sea foods (AAR=19.69%, *p* <0.05), and family history (AAR=27.41%, *p* <0.01) were also significant in conditional logistic regression.	
Fernandez et al.[294]	Hospital-based case-control Gastric cancer cases, n=745 Noncancer controls, n=7990	In-person food frequency questionnaire and lifestyle habits interview	There was a significant inverse trend between fish consumption and gastric cancer odds (OR=0.7, 95%CI 0.5—0.8, 2+ servings/w vs. 1 serving/w; OR=0.8, 95%CI 0.7—0.9, continuous per 1 serving/w, *p* <0.05).	Series of studies that included multiple cancer sites
Ward and Lopez-Carrillo[295]	Population-based case-control Gastric cancer cases, n=220 Population controls, n=752	Food frequency questionnaire	There were significant positive associations between fresh meat (OR=3.1, 95%CI 1.6—6.2, 9+ times/w vs. <4 times/w, *p* for trend = 0.001), processed meat (OR=3.2, 95%CI 1.5—6.6, 6+ times/w vs. <1 time/w, *p* for trend = 0.002), dairy products (OR=2.7, 95%CI 1.4—5.0, 17+ times/w vs. <5 times/w, *p* for trend = 0.003), and fish (OR=2.2, 95%CI 1.2—3.8, 6+ times/w vs. <1 time/w, *p* for trend = 0.001) intakes and gastric cancer odds. These associations were significant for intestinal but not diffuse types of gastric cancer, except for fish intake, which was not significant in either. There were significant negative associations between all vegetables (OR=0.3, 95%CI 0.1—0.6, 6+ times/d vs. <4 times/d, *p* for trend = 0.001), yellow/orange vegetables	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			(OR=0.2, 95%CI 0.1—0.3, 15+ times/w vs. <9 times/w, *p* for trend <0.001), and bean (OR=0.2, 95%CI 0.1—0.3, 7+ times/w vs. ≤1 times/w, *p* for trend <0.001) consumption and gastric cancer odds, which were significant for both intestinal and diffuse types. There was a borderline negative association with citrus fruit consumption (OR=0.7, 95%CI 0.3—1.5, 9+ times/w vs. <2 times/w, *p* for trend = 0.07). There was a significant positive association between salt snacks/cracker consumption frequency and gastric cancer odds (OR=1.8, 95%CI 1.2—2.8, >2 times/m vs. never, *p* for trend = 0.008), which was also significant for intestinal gastric cancer. There was a borderline positive trend for soda and sweets and gastric cancer risk (OR=1.7, 95%CI 0.9—3.2, 15+ times/w vs. <6 times/w, *p* for trend = 0.06).	
Mayne et al.[296]	Population-based case-control Gastric cardia cases, n=255 Gastric non-cardia cases, n=352 Population controls, n=687	In-person interview and food frequency questionnaire 24% of cases' and 33% of controls' blood tested for *H. pylori* but results not included in this analysis due to missing data	Significant positive association with total saturated fat (OR=1.51, 95%CI 1.04—2.19, 75th vs. 25th percentile), dietary sodium (OR=1.46, 95%CI 1.00—2.15, 75th vs. 25th percentile), and nitrites (OR=1.64, 95%CI 1.30—2.07, 75th vs. 25th percentile) and risk of gastric non-cardia cancer. Significant association with sodium not present after adjustment for nitrites. Significant positive associations for total starch (cardia: OR=1.61, 95%CI 1.14—2.28, 75th vs. 25th percentile; non-cardia: OR=2.07, 95%CI 1.51—2.83, 75th vs. 25th percentile), total protein (cardia: OR=1.64, 95%CI 1.11—2.42, 75th vs. 25th percentile; non-cardia: OR=1.52, 95%CI 1.08—2.15, 75th vs. 25th percentile), animal protein (cardia: OR=1.60, 95%CI 1.19—2.15, 75th vs. 25th percentile; non-cardia: OR=1.58, 95%CI 1.22—2.06, 75th vs. 25th percentile), cholesterol (cardia: OR=1.50, 95%CI 1.19—1.90, 75th vs. 25th percentile; non-cardia: OR=1.68, 95%CI 1.35—2.09, 75th vs. 25th percentile), and vitamin B12 (cardia: OR=1.27, 95%CI 1.01—1.60, 75th vs. 25th percentile; non-cardia: OR=1.38, 95%CI 1.13—1.68, 75th vs. 25th percentile) and all gastric cancers. Significant negative associations for PUFA (OR=0.66, 95%CI	"Proxy" interviews for ~30% of cases (protocol not defined). Also evaluated esophageal cancer risk.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			0.47—0.93, 75th vs. 25th percentile) and vegetable protein (OR=0.63, 95%CI 0.45—0.87, 75th vs. 25th percentile) and non-cardia cancer. Significant negative association for dietary fiber and all gastric cancers (cardia: OR=0.43, 95%CI 0.30—0.61, 75th vs. 25th percentile; non-cardia: OR=0.38, 95%CI 0.28—0.53, 75th vs. 25th percentile). Increasing beta-carotene (cardia: OR=0.46, 95%CI 0.34—0.62, 75th vs. 25th percentile; non-cardia: OR=0.58, 95%CI 0.46—0.75, 75th vs. 25th percentile), folate (cardia: OR=0.73, 95%CI 0.55—0.97, 75th vs. 25th percentile; non-cardia: OR=0.67, 95%CI 0.51—0.88, 75th vs. 25th percentile), vitamin C (cardia: OR=0.64, 95%CI 0.49—0.84, 75th vs. 25th percentile; non-cardia: OR=0.59, 95%CI 0.45—0.76, 75th vs. 25th percentile), and vitamin B6 (cardia: OR=0.65, 95%CI 0.47—0.88, 75th vs. 25th percentile; non-cardia: OR=0.59, 95%CI 0.45—0.79, 75th vs. 25th percentile) intake was associated with a significant decreased risk for all stomach cancers. Vitamin E intake was inversely associated with significantly decreased risk of non-cardia gastric cancer (OR=0.71, 95%CI 0.54—0.94, 75th vs. 25th percentile) (borderline significant for cardia (OR=0.75, 95%CI 0.55—1.02, 75th vs. 25th percentile). Supplemental vitamin C inversely associated with gastric non-cardia cancer risk (OR=0.60, 95%CI 0.41—0.88, used supplement 1+ times/w for 6 or more mos).	
Palli et al.[297]	Population-based case-control Gastric cancer cases, n=382 Population controls, n=561	In-person interview and food frequency questionnaire	Significant positive associations for total protein (OR=1.7, 95%CI 1.2—2.5, highest vs. lowest tertile, *p* for trend = 0.002), sodium (OR=1.7, 95%CI 1.2—2.4, highest vs. lowest tertile, *p* for trend = 0.002), and nitrite (OR=1.4, 95%CI 1.0—2.0, highest vs. lowest tertile, *p* for trend = 0.04) and gastric cancer risk. Significant negative associations for vegetable fat (OR=0.7, 95%CI 0.5—1.0, highest vs. lowest tertile, *p* for trend = 0.04), linoleic acid (OR=0.7, 95%CI 0.6—1.0, highest vs. lowest tertile, *p* for trend = 0.04), sugar (OR=0.6, 95%CI 0.4—0.8,	Nitrates are good indicators of vegetable intake.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			highest vs. lowest tertile, *p* for trend = 0.002), vitamin C (OR=0.6, 95%CI 0.4—0.8, highest vs. lowest tertile, *p* for trend = 0.001), beta-carotene (OR=0.6, 95%CI 0.4—0.8, highest vs. lowest tertile, *p* for trend = 0.0009), vitamin E (OR=0.4, 95%CI 0.3—0.7, highest vs. lowest tertile, *p* for trend = 0.0001), and nitrates (OR=0.6, 95%CI 0.4—0.9, highest vs. lowest tertile, *p* for trend = 0.01) and gastric cancer risk. Borderline inverse association for PUFA (OR=0.7, 95%CI 0.5—1.0, highest vs. lowest tertile, *p* for trend = 0.08). From factor analysis, traditional diet significantly associated with increased (OR=3.0, 95%CI 1.8—4.8, highest vs. lowest tertile, *p* for trend = 0.0001) but vitamin-rich diet significantly associated with decreased risk (OR=0.5, 95%CI 0.4—0.7, highest vs. lowest tertile, *p* for trend = 0.0003) of gastric cancer.	
Chen et al.[298]	Population-based case-control Distal gastric cancer cases, n=124 Population controls, n=449	Telephone interview and food frequency questionnaire	Significant inverse associations for vitamin C (OR=0.6, 95%CI 0.3—1.2, highest vs. lowest quartile, *p* for trend = 0.04), carbohydrate (OR=0.4, 95%CI 0.2—0.8, highest vs. lowest quartile, *p* for trend = 0.004), and fiber (OR=0.4, 95%CI 0.2—0.8, highest vs. lowest quartile, *p* for trend = 0.007) intake and the risk of distal gastric cancer. Non-significant negative association with β-cryptoxanthin and gastric cancer risk (OR=0.6, 95%CI 0.3—1.2, highest vs. lowest quartile, *p* for trend = 0.08). There was a significant positive association between saturated fat intake and gastric cancer risk among self-respondents but not among proxy interviewees (OR=3.6, 95% CI 1.0—12.0, highest vs. lowest tertile, *p* for trend =0.03). Use of any vitamin supplement significant associated with a decreased risk of gastric cancer (OR=0.6, 95%CI 0.3—1.0).	80% of gastric cancer cases assessed via proxy interviews of spouses, children, parents or other relatives or friends. No definition for distal stomach cancer, presumably all stomach cancer sites. Also evaluated esophageal cancer risk.
Ito et al.[299]	Hospital-based case-control of women only Gastric cancer cases, n=508	Self-administered lifestyle and food frequency questionnaire	Significant inverse associations between raw vegetables (OR=0.68, 95%CI 0.42—0.82, everyday vs. less than everyday), fruit (OR=0.71, 95%CI 0.59—0.85, everyday vs. less than everyday), green vegetables (OR=0.81, 95%CI 0.68—0.97, 3+ times/w vs. <3 times/w), and carrot (OR=0.73,	*H. pylori* shown to affect risks of differentiated and undifferentiated gastric cancers

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	Non-cancer outpatient controls, n=36,490		95%CI 0.61—0.87, 3+ times/w vs. <3 times/w) intakes and gastric cancer odds. Significant inverse association between cooked fish intake and all types (OR=0.60, 95%CI 0.40—0.90, highest vs. lowest quartile, *p* for trend <0.05) and non-differentiated (OR=0.44, 95%CI 0.26—0.77, highest vs. lowest quartile, *p* for trend <0.005) gastric cancers.	equally.
Lee et al.[300]	Hospital-based case-control Early gastric cancer cases, n=69 Healthy patient controls, n=199	In-person interview and food frequency questionnaire Serum *H. pylori* by ELISA	Family history of gastric cancer (OR=11.6, 95%CI 4.3—31.8, yes vs. no), *H. pylori* infection (OR=5.3, 95%CI 1.7—16.5, yes vs. no), and preference for high salty taste significantly associated with an increased risk of early gastric cancer (*p* <0.01). Significant trend for increased gastric cancer risk with increasing number of cigarettes smoked daily (OR=3.1, 95% CI 1.0—9.5, 21+ cigarettes/d vs. none). Significant trends for decreased gastric cancer risk with increasing clear broth (low salt but no concentration given) (OR=0.2, 95%CI 0.1—0.8, >3 times/w vs. <1 time/w, *p* for trend = 0.01), raw vegetables (OR=0.2, 95%CI 0.1—0.5, >6 times/w vs. <4/w), fruits (OR=0.3, 95%CI 0.1—0.7, >5 times/w vs. <3/w), fruit or vegetable juice (OR=0.6, 95%CI 0.2—1.2, >9 times/mo vs. <2/mo, *p* for trend <0.01), and soybean curd (OR=0.3, 95%CI 0.2—0.8, 1+ times/mo vs. <1/mo). Significant trends for increased gastric cancer risk with more frequent salt-fermented fish (OR=2.4, 95%CI 1.0—5.7, 1+ times/mo vs. <1/mo) and kimchi intakes (OR=1.9, 95%CI 1.3—2.8, 2+ times/d vs. <2/d). Significant interaction between *H. pylori* infection and preferences for salty food and the risk of gastric cancer (*p* = 0.047). Among *H. pylori* positive subjects, preference for salty foods (>0.3% NaCl) conferred increased odds for gastric cancer (OR=10.1, 95%CI 3.4—30.0). *H. pylori* positive subjects with salty preference ≤0.3% NaCl did not have significantly increased odds for gastric cancer (OR=1.7,	Definition of early gastric cancer included patients newly diagnosed without previous gastric surgery, no mention of stage/grade.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			95%CI 0.6—4.7).	
Kim et al.[301]	Hospital-based case-control Gastric cancer cases, n=136 Surgical/clinic patient controls, n=136	In-person interview and food frequency questionnaire Serum *H. pylori* by ELISA	Significant inverse associations between dietary fiber (OR=0.37, 95%CI 0.17—0.79, highest vs. lowest tertile, *p* for trend = 0.009), vegetable protein (OR=0.39, 95%CI 0.18—0.82, highest vs. lowest tertile, *p* for trend = 0.013), calcium (OR=0.43, 95%CI 0.21—0.90, highest vs. lowest tertile, *p* for trend = 0.025), phosphorus (OR=0.38, 95%CI 0.16—0.88, highest vs. lowest tertile, *p* for trend = 0.025), potassium (OR=0.36, 95%CI 0.17—0.77, highest vs. lowest tertile, *p* for trend = 0.007), vitamin A (OR=0.36, 95%CI 0.17—0.77, highest vs. lowest tertile, *p* for trend = 0.008), β-carotene (OR=0.35, 95%CI 0.16—0.75, highest vs. lowest tertile, *p* for trend = 0.007), thiamin (OR=0.42, 95%CI 0.19—0.91, highest vs. lowest tertile, *p* for trend = 0.039), riboflavin (OR=0.37, 95%CI 0.18—0.77, highest vs. lowest tertile, *p* for trend = 0.007), vitamin B6 (OR=0.35, 95%CI 0.15—0.80, highest vs. lowest tertile, *p* for trend = 0.009), vitamin E (OR=0.48, 95%CI 0.24—0.98, highest vs. lowest tertile, *p* for trend = 0.037), and folate (OR=0.40, 95%CI 0.20—0.83, highest vs. lowest tertile, *p* for trend = 0.014) intakes and gastric cancer risk in adjusted model (includes adjustment for *H. pylori* infection). Vegetable fat inverse trend borderline significant, but tertile ORs significant (OR=0.49, 95%CI 0.24—0.99, highest vs. lowest tertile, *p* for trend = 0.055). Riboflavin (OR=0.42, 95%CI 0.21—0.89, highest vs. lowest tertile, *p* for trend = 0.021) and vitamin C (OR=0.35, 95%CI 0.17—0.74, highest vs. lowest tertile, *p* for trend = 0.006) also significantly associated with reduced gastric cancer risk when dietary intake combined with supplement use. Significant protective effects of vitamins E (OR=0.16, 95%CI 0.03—0.83, highest vs. lowest tertile, *p* for trend = 0.028) and C (OR=0.10, 95%CI 0.02—0.63, highest vs. lowest tertile, *p* for trend = 0.015) were evident among *H. pylori*-positive subjects. Interaction not statistically significant.	Effect of vegetable fat not significant when adjusted for vitamin E. Study population consumed relatively high amounts of PUFA and MUFA compared to saturated fats. Dietary antioxidants may inhibit the formation of *N*-nitroso compounds from secondary amines and nitrites in the stomach by reducing nitrites to nitric oxide or modifying amines.
Phukan et	Hospital-based	In-person interview and food	Significant positive trends with smoked dried salted fish	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
al.[302]	case-control Gastric cancer cases, n=329 Non-cancer patient controls, n=665	frequency questionnaire Spot urease from biopsy sample (for *H. pylori*) Serum *H. pylori* by ELISA	(OR=2.8, 95%CI 1.8—8.4, 2+ times/w vs. never, *p* for trend <0.001) and meat (OR=2.8, 95%CI 1.7—8.8, 2+ times/w vs. never, *p* for trend <0.001) consumption and gastric cancer odds. Significant positive trends also seen with sa-um (fermented pork fat) (OR=3.4, 95%CI 1.7—10.3, 2+ times/w vs. never, *p* for trend <0.001) and soda (OR=2.9, 95%CI 1.2—6.5, 2+ times/w vs. never, *p* for trend <0.001) added as a food preservative. Significant interaction between *H. pylori* infection and smoked salted meat (OR=1.9, *p* <0.046) or sa-um (OR=2.1, *p* <0.02) intake and gastric cancer risk.	
Kuriki et al.[303]	Hospital-based case-control Gastric cancer cases, n=179 Outpatient controls, n=357	In-person interview and food frequency questionnaire Erythrocyte fatty acid composition	Significantly increased odds for gastric cancer with increasing sodium intake (poorly differentiated: OR=1.97, 95%CI 1.19—3.25, highest vs. lowest tertile, *p* for trend <0.05; well-differentiated: OR=2.67, 95%CI 0.97—5.79, *p* for trend = 0.06). Sodium intake positively correlated to fish (r = 0.41, *p* <0.0001) and seafood (r = 0.45, *p* <0.0001) intake but not erythrocyte PUFAs. Significant positive associations between erythrocyte saturated fat (OR=2.3, 95%CI 1.39—3.90), highest vs. lowest tertile, *p* for trend <0.005) and palmitic acid (OR=3.14, 95%CI 1.77—5.70, highest vs. lowest tertile, *p* for trend <0.001) composition and gastric cancer odds. Significant inverse associations between erythrocyte DHA (OR=0.47, 95%CI 0.28—0.79, highest vs. lowest tertile, *p* for trend <0.01), docosapentaenoic acid (OR=0.32, 95%CI 0.18—0.56, highest vs. lowest tertile, *p* for trend = 0.0005) and total n-3 highly unsaturated fatty acids (at least 20 carbons, OR=0.39, 95%CI 0.23—0.68, highest vs. lowest tertile, *p* for trend <0.005) and gastric cancer odds.	
Wu et al.[304]	Population-based case-control Gastric cardia cancer cases, n=257	In-person interview and food frequency questionnaire	Gastric cardia cancer odds significantly increased with increasing total fat (OR=1.44, 95%CI 0.9—2.2, highest vs. lowest quartile, *p* for trend = 0.046), saturated fat (OR=1.52, 95%CI 1.0—2.4, highest vs. lowest quartile, *p* for trend = 0.004), and MUFA (OR=1.31, 95%CI 0.9—2.0, highest vs.	Also evaluated esophageal cancer risk. *H. pylori* results for 33-50% of subjects

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	Distal gastric cancer cases, n=366 Population controls, n=1308		lowest quartile, *p* for trend = 0.08) intakes. Distal gastric cancer odds significantly increased with increasing cholesterol intake (OR=2.39, 95%CI 1.6—3.5, highest vs. lowest quartile, *p* for trend <0.001). Carbohydrate intake significantly inversely associated with gastric cancer odds (cardia: OR=0.58, 95%CI 0.4—0.9, highest vs. lowest quartile, *p* for trend = 0.016; distal: OR=0.58, 95%CI 0.4—0.9, highest vs. lowest quartile, *p* for trend = 0.003). Highest vs. lowest fiber intake significantly protective for all gastric cancers (cardia: OR=0.58, 95%CI 0.4—0.9; non-cardia: OR=0.69, 95%CI 0.5—1.0, *p* for trend = 0.15), but quartile trend significant in cardia cancer only (*p* for trend = 0.016). Association with total fat no longer present when adjusting for fiber. Gastric cancer odds significantly increased with increasing meat (including fish) intake (cardia: OR=1.67, 95%CI 1.03—2.7, highest vs. lowest quartile, *p* for trend = 0.027; distal: OR=1.91, 95% CI 1.3—2.9, highest vs. lowest quartile, *p* for trend = 0.004). Significant positive trends for red meat and gastric cardia cancer risk (OR=1.56, 95%CI 0.97—2.5, highest vs. lowest quartile, *p* for trend = 0.031) and processed meat and distal gastric cancer risk (OR=1.65, 95%CI 1.1—2.5, highest vs. lowest quartile, *p* for trend = 0.049).	from previous analysis did not impact fiber, fat, and cholesterol results.
Hu et al.[305]	Population-based case-control Gastric cancer cases, n=1182 Population controls, n=5039	Mailed questionnaire with food frequency questionnaire	Total meat (OR=1.6, 95%CI 1.3—2.1, highest vs. lowest quartile, *p* for trend = 0.0001), processed meat (OR=1.7, 95%CI 1.3—2.2, highest vs. lowest quartile, *p* for trend = 0.0001), and total fish (OR=1.3, 95% CI 1.0—1.6, *p* for trend = 0.05) consumption significantly associated with increased gastric cancer odds. High poultry intake was significantly associated with decreased gastric cancer odds in men only (OR=0.7, *p* for interaction <0.05).	
Navarro Silvera et al.[306]	Population-based case-control Gastric cardia	In-person interview and food frequency questionnaire	All meats (OR=1.37, 95%CI 1.08—1.73, continuous based on increase of 1 serving/d), poultry (OR=1.89, 95%CI 1.15—3.11, continuous based on increase of 1 serving/d), grains	Also evaluated esophageal cancers.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	cancer cases, n=255 Gastric non-cardia cancer cases, n=352 Population controls, n=687		(OR=1.20, 1.02—1.42, continuous based on increase of 1 serving/d), and high-fat dairy (OR=1.23, 95%CI 1.01—1.51, continuous based on increase of 1 serving/d) significantly associated with increased gastric cardia cancer odds. All meats (OR=1.39, 95%CI 1.12—1.71, continuous based on increase of 1 serving/d), poultry (OR=1.90, 95%CI 1.19—3.03, continuous based on increase of 1 serving/d), high-nitrite meats (OR=1.88, 95%CI 1.24—2.84, continuous based on increase of 1 serving/d), grains (OR=1.36, 95%CI1.17—1.59, continuous based on increase of 1 serving/d), and refined grains (OR=1.51, 95%CI 1.25—1.82, continuous based on increase of 1 serving/d) significantly associated with increased gastric non-cardia cancer odds. Borderline significant inverse association between non-citrus fruits and gastric cardia cancer risk (OR=0.84, 95%CI 0.68—1.03, continuous based on increase of 1 serving/d). Significantly decreased risk for gastric non-cardia cancer with increasing low-fat dairy intake (OR=0.60, 95%CI 0.41—0.88, continuous based on increase of 1 serving/d).	
Lucenteforte et al.[307]	Hospital-based case-control Gastric cancer cases, n=230 Non-cancer patient controls, n=547	In-person interview and food frequency questionnaire	Significant positive trend with total energy intake and gastric cancer odds (OR=1.79, 95% CI 1.16—2.76, highest vs. lowest tertile, *p* for trend = 0.01). Significant inverse associations with total vegetable fats (OR=0.65, 95%CI 0.43—0.98, highest vs. lowest tertile, *p* for trend = 0.06), PUFA (OR=0.66, 95%CI 0.44—0.97, highest vs. lowest tertile, *p* for trend = 0.03), and linoleic acid (OR=0.67, 95%CI 0.45—1.00, highest vs. lowest tertile, *p* for trend = 0.05) and gastric cancer odds.	
Pourfarzi et al.[308]	Population-based case-control Gastric cancer cases, n=217 Population controls, n=394	In-person interview and dietary questionnaire Serum *H. pylori* by ELISA	Significant increased odds for gastric cancer with positive *H. pylori* status (OR=1.72, 95%CI 1.12—2.63, positive vs. negative). Daily consumption of red meats (OR=3.40, 95%CI 1.76—6.46, at least once daily vs. 2 or fewer time/w, *p* for trend <0.01) and dairy products (OR=2.28, 95%CI 1.23—4.22, at least once daily vs. 2 or fewer times/w, *p* for trend <0.01)	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			significantly increased gastric cancer odds. Allium vegetables (garlic (OR=0.35, 95%CI 0.13—0.95, 3 or more times/w vs. never or infrequently, *p* for trend <0.01) and onions (OR=0.34, 95%CI 0.019—0.62, at least once daily vs. 2 or fewer times/w, *p* for trend = 0.02)), fresh fruits (OR=0.45, 95%CI 0.29—0.68, at least 3 times/w vs. never or infrequently, *p* for trend <0.01), citrus fruits (OR=0.31, 95%CI 0.17—0.59, at least 3 times/w vs. never or infrequently, *p* for trend <0.01), and fresh fish (OR=0.37, 95%CI 0.19—0.70, at least once weekly vs. never or infrequently) consumption significantly inversely associated with gastric cancer odds. Significantly increased risk for gastric cancer with hot (OR=2.85, 95%CI 1.65—4.91, hot vs. not hot) and strong (OR=2.64, 95%CI 1.45—4.80, strong vs. not strong) tea consumption and preference for high salt intake (OR=3.10, 95%CI 1.88—5.10, salty vs. not salty). These results (except fresh fruits) include adjustment for *H. pylori* status and salt preference.	
Sumathi et al.[309]	Hospital-based case-control Gastric cancer cases, n=89 Non-cancer dyspeptic controls, n=89	In-person interview and food frequency questionnaire	Significant positive associations between alcohol consumption (OR=2.3, 95%CI 1.1—4.9, *p* = 0.04) and pickled foods (OR=1.8, 95%CI 1.2—3.9, *p* = 0.05) and gastric cancer odds. Significant inverse association between pulses (legumes, OR=0.4, 95%CI 0.2—0.9, *p* = 0.05) and gastric cancer odds.	
Pakseresht et al.[310]	Population-based case-control Gastric cancer cases, n=286 Population controls, n=304	In-person interview and food frequency questionnaire Serum *H. pylori* and CagA via Western blot	No significant difference in proportion of *H. pylori*-positive subjects in cases vs. controls (both groups at least 97% positive, *p* = 0.16). Cases significantly more likely to be CagA positive (*p* = 0.04). Significant inverse associations between protein (OR=0.87, 95%CI 0.76—0.99, per 10 g), vitamin C (OR=0.82, 95%CI 0.76—0.87, per 10 mg), zinc (OR=0.47, 95%CI 0.32—0.70, per 5 mg), and iron (OR=0.37, 95%CI 0.32—0.70, per 5 mg) intakes and gastric cancer odds, with adjustment for *H. pylori* status.	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			Significant positive association for total fat and gastric cancer odds, with adjustment for *H. pylori* status (OR=1.33, 95%CI 1.12—1.57, per 20 g)	
Icli et al.[311]	Hospital-based case-control Gastric cancer cases, n=253 Friends and relatives of cases as controls, n=253	In-person interview and food frequency questionnaire	Significant inverse associations for salad (OR=0.2, 95%CI 0.1—0.7, highest vs. lowest tertile, *p* for trend = 0.006), coffee (OR=0.3, 95% CI 0.2—0.7, highest vs. lowest tertile, *p* for trend <0.0001), tomatoes and cucumbers at breakfast (OR=0.6, 95%CI 0.4—0.9, preference versus no preference, *p* = 0.009), and fish (OR=0.6, 95% CI 0.4—1.0, preference vs. no preference, *p* = 0.036) and dried legumes (OR=0.7, 95%CI 0.5—1.0, preference vs. no preference, *p* = 0.043) at lunch and dinner and gastric cancer odds in multivariate model. Significant positive association between animal-derived cooking oil use and gastric cancer odds in multivariate model (OR=1.9, 95% CI 1.2—3.0, animal vs. plant oil, *p* = 0.003).	

The association between fat intake and gastric cancer risk appears to be complex. Kabat et al.[290] reported a significant positive trend for gastric cardia cancer risk with increasing fat intake (OR = 2.9, 95% CI 1.5—5.6, highest versus lowest quartile, *p* for trend <0.001). However, subsequent studies detected inverse associations between fat, particularly plant fats, and gastric cancer risk. Ji et al.[291] demonstrated significantly decreased odds for gastric cancer with increasing fat intake among women (OR = 0.6, 95% CI 0.4—0.8, highest versus lowest quartile, *p* for trend = 0.006) and with increasing plant oil intake among both sexes (men: OR = 0.7, 95% CI 0.5—0.9, *p* for trend = 0.001; women: OR = 0.6, 95% CI 0.4—1.0, *p* for trend = 0.01, highest versus lowest quartile). The results of Kaaks et al.[292] show a significant reduction in gastric cancer risk with increasing intake of LA (OR = 0.53, highest versus lowest quartile, *p* for trend <0.001) and borderline inverse associations for MUFA (OR = 0.75, highest versus lowest quartile, *p* for trend = 0.064) and PUFA (OR = 0.69, highest versus lowest quartile, *p* for trend = 0.065). Additionally, Palli et al.[297] concluded that increased consumption of vegetable fat (OR = 0.7, 95% CI 0.5—1.0, highest versus lowest tertile, *p* for trend = 0.04) and LA (OR = 0.7, 95% CI 0.6—1.0, highest versus lowest tertile, *p* for trend = 0.04) conferred a significant 30% reduction in gastric cancer risk, while PUFA were borderline significantly inversely associated with gastric cancer risk (OR = 0.7, 95% CI 0.5—1.0, highest versus lowest tertile, *p* for trend = 0.08). Kim et al.[301] reported a significant decrease in gastric cancer odds with increased vegetable fat intake (OR = 0.49, 95% CI 0.24—0.99, highest versus lowest tertile, *p* for trend = 0.055), but this association was no longer present when adjusting for vitamin E, suggesting that the protective effect was due

to the vitamin E present in the vegetable fat. Inverse associations for total vegetable fats (OR = 0.65, 95% CI 0.43—0.98, highest versus lowest tertile, *p* for trend = 0.06), PUFA (OR = 0.66, 95% CI 0.44—0.97, highest versus lowest tertile, *p* for trend = 0.03), and linoleic acid (OR = 0.67, 95% CI 0.45—1.00, highest versus lowest tertile, *p* for trend = 0.05) and gastric cancer odds were also detected by Lucenteforte et al.[307].

Mayne et al.[296] reported a significant positive association between saturated fat and gastric non-cardia cancer odds (OR = 1.51, 95% CI 1.04—2.19, 75^{th} versus 25^{th} percentile), while PUFA were significantly inversely associated with this cancer site (OR = 0.66, 95% CI 0.47—0.93, 75^{th} versus 25^{th} percentile). Significant positive associations for total fat (OR = 1.44, 95% CI 0.9—2.2, highest versus lowest quartile, *p* for trend = 0.046), saturated fat (OR = 1.52, 95% CI 1.0—2.4, highest versus lowest quartile, *p* for trend = 0.004), and MUFA (OR = 1.31, 95% CI 0.9—2.0, highest versus lowest quartile, *p* for trend = 0.08) and gastric cardia cancer odds were demonstrated by Wu et al.[304]; however, the association for total fat was no longer significant when adjusting for fiber intake. Subsequently, Navarro Silvera et al.[306] concluded that high-fat dairy intake was significantly positively associated with gastric cardia cancer odds (OR = 1.23, 95% CI 1.01—1.51, based on an increase of one serving per day). A recent study by Pakseresht et al.[310] reported a significant positive association between total fat intake and gastric cancer odds (OR = 1.33, 95% CI 1.12—1.57, per each 20 g increase in intake). In addition to subjective dietary data, Kuriki et al.[303] evaluated erythrocyte fatty acid composition and demonstrated significant positive associations between concentration of saturated fat (OR = 2.3, 95% CI 1.39—3.90, highest versus lowest

tertile, *p* for trend <0.0005) and palmitic acid (OR = 3.14, 95% CI 1.77—5.70, highest versus lowest tertile, *p* for trend <0.001) and gastric cancer odds and significant negative associations between DHA (OR = 0.47, 95% CI 0.28—0.79, highest versus lowest tertile, *p* for trend <0.01) and total highly-unsaturated n-3 (at least 20 carbons, OR = 0.39, 95% CI 0.23—0.68, highest versus lowest tertile, *p* for trend <0.005) and gastric cancer odds. Although case-control studies conducted to date indicate that high total fat and saturated fat intakes may increase the risk of both cardia and non-cardia gastric cancer, evidence suggests an inverse association between vegetable fats, MUFA, PUFA, n-3, and LA and gastric cancer risk.

Similar to the results seen with fat intake, the association between fish intake and gastric cancer risk evaluated via case-control studies is equivocal. In a series of case-control studies of multiple cancer sites, Fernandez et al.[294] concluded that fish consumption at least twice weekly significantly decreased gastric cancer odds by 30% compared to fish consumption once per week (OR = 0.7, 95% CI 0.5—0.8). More recently, Pourfarzi et al.[308] detected a significant inverse association between fresh fish consumption and gastric cancer odds (OR = 0.37, 95% CI 0.19—0.70, at least once weekly versus never or infrequently), and Ito et al.[299] measured significantly decreased gastric cancer odds with increasing intake of cooked fish in a case-control study of women only (OR = 0.60, 95% CI 0.40—0.90, highest versus lowest quartile, *p* for trend <0.05). Icli et al.[311] calculated significantly decreased gastric cancer odds with fish intake at lunch and dinner (OR = 0.6, 95% CI 0.4—1.0, preference versus no preference, *p* = 0.036). Conversely, several studies have linked fish intake to increased gastric cancer

odds. Ward and Lopez-Carrillo[295] concluded that total fish intake more than doubled gastric cancer odds (OR = 2.2, 95% CI 1.2—3.8, at least six servings per week versus less than one serving per week, *p* for trend = 0.001). Additionally, Hu et al.[305] showed that increasing total fish consumption was significantly associated with a moderate increase in gastric cancer odds (OR = 1.3, 95% CI 1.0—1.6, highest versus lowest quartile, *p* for trend = 0.05).

In contrast, case-control studies that have examined the intake of salted or fermented fish products have tended to show a positive association between the consumption of such products and gastric cancer risk. Ye et al.[293] showed that fish sauce (OR = 2.57, 95% CI 1.89—3.50, at least 0.4 kg/month versus less than 0.4 kg/month, $p < 0.01$) and salted, fermented sea foods (OR = 1.57, 95% CI 1.21—2.02, at least 1.5 kg/year versus less than 1.5 kg/year, $p < 0.01$) were significantly associated with increased odds of gastric cancer. Conditional logistic regression models demonstrated that fish sauce and salted sea foods were attributable for 17.81% ($p < 0.01$) and 19.69% ($p < 0.05$) of gastric cancer cases, respectively. In a smaller study, Lee et al.[300] reported that monthly consumption of salt-fermented fish significantly increased gastric cancer risk compared to less than monthly intake (OR = 2.4, 95% CI 1.0—5.7). Phukan et al.[302] demonstrated that frequent intake of smoked salted fish nearly tripled gastric cancer odds (OR = 2.8, 95% CI 1.8—8.4, at least weekly versus never, *p* for trend < 0.001). When considered with the evidence from the ecological study previously reviewed, it appears likely that fermented fish products—fish sauce, in particular—increase the risk of gastric cancer when consumed frequently. However, more accurate

assessment of the types of fish consumed and preparation techniques employed are needed to further characterize the associations between fish consumption and gastric cancer risk among the general population.

Similar associations were seen regarding fruit and vegetable intake in case-control studies; fresh fruits and vegetables have been linked to decreased risk for gastric cancer, while pickled or preserved vegetables (which are high in salt) show an increased risk of gastric cancer with increasing consumption. Ji et al.[291] concluded that preserved vegetable intake significantly increases the risk of gastric cancer in women only (OR = 1.9, 95% CI 1.3—2.8, highest versus lowest quartile, *p* for trend = 0.002) but demonstrated significant inverse associations between all vegetables (OR = 0.4, 95% CI 0.3—0.5, highest versus lowest quartile, *p* for trend <0.0001), yellow or green vegetables (OR = 0.5, 95% CI 0.4—0.7, highest versus lowest quartile, *p* for trend = 0.0001), and soybean products (OR = 0.5, 95% CI 0.4—0.7, highest versus lowest quartile, *p* for trend = 0.0001) and gastric cancer risk among the males participating in the study. A significant inverse trend was also observed for fresh fruit intake and gastric cancer risk among both genders (men: OR = 0.4, 95% CI 0.3—0.6, *p* for trend <0.0001; women: OR = 0.5, 95% CI 0.3—0.8, highest vs. lowest, *p* for trend = 0.0006). A subsequent study by Ye et al.[293] showed a significant reduction in gastric cancer risk with more frequent consumption of citrus (OR = 1.41, 95% CI 1.03—1.92, ≤2.5 kg per year versus >2.5 kg/y, *p* <0.05) and other fruits (OR = 1.31, 95% CI 1.01—1.71, ≤2.5 kg per year versus >2/5 kg per year, *p* <0.05) and fresh vegetables (OR = 1.95, 95% CI 1.41—2.70, ≤25 kg per year versus >25 kg per year, *p* <0.01) and a significant increase in risk with

consumption of salted vegetables (OR = 1.41, 95% CI 1.09—1.83, ≥2 kg per year versus <2 kg per year, p <0.05), but these results were not adjusted for important factors such as age, gender, and total energy intake. All vegetables (OR = 0.3, 95% CI 0.1—0.6, at least six times daily versus less than four times daily, p for trend = 0.001) and yellow or orange vegetables specifically (OR = 0.2, 95% CI 0.1—0.3, at least 15 servings weekly versus fewer than nine servings weekly, p for trend <0.001) were significantly and negatively associated with gastric cancer odds in a study by Ward and Lopez-Carrillo[295], which also detected a borderline inverse association between citrus fruit consumption and gastric cancer odds (OR = 0.7, 95% CI 0.3—1.5, at least nine servings weekly versus less than two servings weekly, p for trend = 0.07). In a case-control study of women only, Ito et al.[299] concluded that increased raw vegetable (OR = 0.68, 95% CI 0.42—0.82, everyday versus less than everyday), fruit (OR = 0.71, 95% CI 0.59—0.85, everyday versus less than everyday), green vegetable (OR = 0.81, 95% CI 0.68—0.97, at least three servings weekly versus less than three servings weekly), and carrot (OR = 0.73, 95% CI 0.61—0.87, at least three servings weekly versus less than three servings weekly) intakes were significantly associated with decreased odds for gastric cancer. Lee et al.[300] measured significant inverse trends for raw vegetables (OR = 0.2, 95% CI 0.1—0.5, more than six times per week versus less than four times per week), fruits (OR = 0.3, 95% CI 0.1—0.7, more than five times per week versus less than three times per week), fruit or vegetable juice (OR = 0.6, 95% CI 0.2—1.2, more than nine times monthly versus less than two times monthly, p for trend <0.01), and soybean curd (OR = 0.3, 95% CI 0.2—0.8, at least monthly versus less than monthly) consumption and

the risk of early gastric cancer in a small case-control study (n=69 cases). A borderline significant inverse association between non-citrus fruit consumption and gastric cardia cancer risk was detected by Navarro Silvera et al.[306] (OR = 0.84, 95% CI 0.68—1.03, with each increase of one serving per day). Pourfarzi et al.[308] concluded that increased consumption of allium vegetables such as garlic (OR = 0.35, 95% CI 0.13—0.95, three or more times per week versus never or infrequently, *p* for trend <0.01) and onions (OR = 0.34, 95% CI 0.019—0.62, at least once daily versus twice or fewer times per week, *p* for trend = 0.02), fresh fruits (OR = 0.45, 95% CI 0.29—0.68, at least three servings per week versus never or infrequently, *p* for trend <0.01), and citrus fruits (OR = 0.31, 95% CI 0.17—0.59, at least three servings per week versus never or infrequently, *p* for trend <0.01) was significantly associated with decreased odds for gastric cancer. More recently, Icli et al.[311] noted a significant inverse association between a preference for consuming tomatoes and cucumbers at breakfast (OR = 0.6, 95% CI 0.4—0.9, *p* = 0.009) and increased frequency of salad consumption (OR = 0.2, 95% CI 0.1—0.7, highest versus lowest tertile, *p* for trend = 0.006) and the risk of gastric cancer in a Turkish case-control study. However, this study used friends and relatives of the cancer cases as control subjects, which may have contributed bias to the results.

When quantifying antioxidant nutrient intakes associated with fruit and vegetable consumption, Ji et al.[291] concluded that increasing vitamin E (men: OR = 0.5, 95% CI 0.3—0.7, highest versus lowest quartile, *p* for trend = 0.0002; women: OR = 0.5, 95% CI 0.3—0.8, highest versus lowest quartile, *p* for trend = 0.0002) and carotene (men: OR = 0.4, 95% CI 0.3—0.6, highest versus lowest quartile, *p* for trend <0.0001; women: OR =

0.7, 95% CI 0.5—1.1, highest versus lowest quartile, *p* for trend = 0.02) intakes significantly decreased the risk for gastric cancer among men and women. A significant inverse trend was also noted for vitamin C among men, with the highest quartile of intake conferring a 50% reduction in odds (OR = 0.5, 95% CI 0.3—0.7, versus lowest quartile, *p* for trend <0.0001). Kaaks et al.[292] also detected significant inverse associations between vitamin C (OR = 0.43, highest versus lowest quartile, *p* for trend <0.001) and β-carotene (OR = 0.50, highest versus lowest quartile, *p* for trend <0.001) and gastric cancer risk. Similar significant inverse associations with β-carotene (cardia: OR = 0.46, 95% CI 0.34—0.62, 75^{th} vs. 25^{th} percentile; non-cardia: OR = 0.58, 95% CI 0.46—0.75, 75^{th} vs. 25^{th} percentile) and vitamin C from supplements (non-cardia: OR = 0.60, 95% CI 0.41—0.88, used supplement at least once weekly for at least six months versus no use) or food (cardia: OR = 0.64, 95% CI 0.49—0.84, 75^{th} versus 25^{th} percentile; non-cardia: OR = 0.59, 95% CI 0.45—0.76, 75^{th} versus 25^{th} percentile) were observed for gastric cancers by Mayne et al.[296], who also noted a significantly decreased risk for non-cardia gastric cancer with increasing vitamin E intake (OR = 0.71, 95% CI 0.54—0.94, 75^{th} versus 25^{th} percentile). This association was borderline significant for cardia gastric cancer (OR = 0.75, 95% CI 0.55—1.02, 75^{th} versus 25^{th} percentile).

In a subsequent study, Palli et al.[297] demonstrated significant inverse associations between vitamin C (OR = 0.6, 95% CI 0.4—0.8, highest versus lowest tertile, *p* for trend = 0.001), β-carotene (OR = 0.6, 95% CI 0.4—0.8, highest versus lowest tertile, *p* for trend = 0.0009), vitamin E (OR=0.4, 95% CI 0.3—0.7, highest versus lowest tertile, *p* for trend = 0.0001), and nitrates (as a marker of vegetable consumption,

OR = 0.6, 95% CI 0.4—0.9, highest versus lowest tertile, *p* for trend = 0.01) and risk of gastric cancer. Vitamin C intake was also shown to be significantly inversely associated with distal gastric cancer risk by Chen et al.[298] (OR = 0.6, 95% CI 0.3—1.2, highest versus lowest quartile, *p* for trend = 0.04); however, the authors did not define distal gastric cancer, and 80% of the interviews for gastric cancer cases were conducted with spouses, children, parents, or other relatives or friends of the subjects due to the high mortality rate among gastric cancer cases.

From a hospital based case-control study, Kim et al.[301] detected significant inverse associations between β-carotene (OR = 0.35, 95% CI 0.16—0.75, highest versus lowest tertile, *p* for trend = 0.007), vitamin E, (OR = 0.48, 95% CI 0.24—0.98, highest versus lowest tertile, *p* for trend = 0.037), and total vitamin C (from supplements and food, OR = 0.35, 95% CI 0.17—0.74, highest versus lowest tertile, *p* for trend = 0.006) intake and gastric cancer risk; these results included adjustment for *H. pylori* infection status. Finally, Pakseresht et al.[310] concluded that vitamin C (OR = 0.82, 95% CI 0.76—0.87, per 10 mg), zinc (OR = 0.47, 95% CI 0.32—0.70, per 5 mg), and iron (OR = 0.37, 95% CI 0.32—0.70, per 5 mg) intakes were significantly inversely associated with gastric cancer risk, with adjustment for *H. pylori*.

Case-control studies have also provided evidence for associations between additional dietary factors, such as alcohol, total energy, protein, carbohydrate, fiber, legume, coffee, and tea consumption, as well as eating habits and dietary patterns and the risk of gastric cancer. Kabat et al.[290] concluded that increasing alcohol consumption, particularly hard liquor, increased the risk of gastric cardia cancer among men (OR = 2.3,

95% CI 1.3—4.3, at least 4 oz. whiskey equivalents per day versus nondrinkers); among women, daily beer consumption significantly increased the odds of gastric cardia cancer by nearly five-fold (OR = 4.9, 95% CI 1.1—22.8, versus nondrinkers). Sumathi et al.[309] also detected a substantial increased risk for gastric cancer with increasing alcohol intake (OR = 2.3, 95% CI 1.1—4.9, $p = 0.04$).

In addition, Kabat et al.[290] reported a significant inverse association between fiber intake and gastric cardia cancer risk (OR = 3.2, 95% CI 1.5—7.0, lowest versus highest quartile, p for trend <0.01). Ji et al.[291] detected significant inverse associations between fiber (men: OR = 0.6, 95% CI 0.4—0.8, highest versus lowest, p for trend = 0.002; women: OR = 0.6, 95% CI 0.3—0.9, highest versus lowest, p for trend = 0.007) and protein intake (men: OR = 0.7, 95% CI 0.5—0.9, highest versus lowest, p for trend = 0.003; women: OR = 0.6, 95% CI 0.4—1.0, highest versus lowest, p for trend = 0.01) and gastric cancer risk and a significant positive trend for carbohydrate intake and gastric cancer risk among both men women (men: OR = 1.5, 95% CI 1.1—2.1, highest versus lowest, p for trend = 0.002; women: OR = 1.9, 95% CI 1.3—2.9, highest versus lowest, p for trend = 0.0007). Kaaks et al.[292] concluded that total protein (OR = 0.64, highest versus lowest quartile, p for trend = 0.024), total vegetable protein (OR = 0.53, highest versus lowest quartile, p for trend = 0.001), total polysaccharides (OR = 0.63, highest versus lowest quartile, p for trend = 0.002), and total dietary fiber (OR = 0.31, highest versus lowest quartile, p for trend <0.001) intakes were significantly inversely associated with gastric cancer odds, while total energy (OR = 2.19, highest versus lowest quartile, p for trend <0.001) and total mono- and disaccharides (OR = 1.88, highest versus lowest

quartile, *p* for trend <0.001) intakes were significantly associated with increased gastric cancer odds. Ward and Lopez-Carrillo[295] detected a significant inverse association between bean consumption and gastric cancer odds (OR = 0.2, 95% CI 0.1—0.3, at least seven servings per week versus one or fewer servings per week, *p* for trend <0.001) but a borderline positive trend for soda and sweets intakes and gastric cancer odds (OR = 1.7, 95% CI 0.9—3.2, 15 or more servings per week versus less than six servings per week, *p* for trend = 0.06). Mayne et al.[296] concluded that increasing total starch (cardia: OR = 1.61, 95% CI 1.14—2.28, 75^{th} versus 25^{th} percentile; non-cardia: OR = 2.07, 95% CI 1.51—2.83, 75^{th} versus 25^{th} percentile), total protein (cardia: OR = 1.64, 95% CI 1.11—2.42, 75^{th} versus 25^{th} percentile; non-cardia: OR = 1.52, 95% CI 1.08—2.15, 75^{th} versus 25^{th} percentile), and animal protein (cardia: OR = 1.60, 95% CI 1.19—2.15, 75^{th} versus 25^{th} percentile; non-cardia: OR = 1.58, 95% CI 1.22—2.06, 75^{th} versus 25^{th} percentile) consumption significantly increased the risk for all gastric cancers while increasing dietary fiber intake significantly decreased the risk for all gastric cancers (cardia: OR = 0.43, 95% CI 0.30—0.61, 75^{th} versus 25^{th} percentile; non-cardia: OR = 0.38, 95% CI 0.28—0.53, 75^{th} versus 25^{th} percentile) and increasing vegetable protein intake significantly decreased the risk for non-cardia gastric cancers (OR = 0.63, 95% CI 0.45—0.87, 75^{th} versus 25^{th} percentile). A significant positive association for total protein intake and gastric cancer risk was also detected by Palli et al.[297] (OR = 1.7, 95% CI 1.2—2.5, highest versus lowest tertile, *p* for trend = 0.002). Chen et al.[298] concluded that carbohydrate (OR = 0.4, 95% CI 0.2—0.8, highest versus lowest quartile, *p* for trend = 0.004) and fiber intakes (OR = 0.4, 95% CI 0.2—0.8, highest versus lowest quartile, *p*

for trend = 0.007) significantly reduced distal gastric cancer odds by 60%; as mentioned previously, this study largely relied on proxy interviews which may have biased the results. Kim et al.[301] demonstrated significant inverse associations between dietary fiber (OR = 0.37, 95% CI 0.17—0.79, highest versus lowest tertile, *p* for trend = 0.009) and vegetable protein (OR = 0.39, 95% CI 0.18—0.82, highest versus lowest tertile, *p* for trend = 0.013) consumption and gastric cancer risk after adjustment for *H. pylori* status. Wu et al.[304] concluded that carbohydrate intake was significantly inversely associated with gastric cancer risk (cardia: OR = 0.58, 95% CI 0.4—0.9, highest versus lowest quartile, *p* for trend = 0.016; distal: OR = 0.58, 95% CI 0.4—0.9, highest versus lowest quartile, *p* for trend = 0.003) and that the highest quartile of fiber intake significantly reduced the risk for all gastric cancers when compared to the lowest quartile (cardia: OR = 0.58, 95% CI 0.4—0.9; non-cardia: OR = 0.69, 95% CI 0.5—1.0, *p* for trend = 0.15), but the inverse trend was significant in gastric cardia cancers only (*p* for trend = 0.016). Navarro Silvera et al.[306] demonstrated a significant positive association between grain intake and gastric non-cardia cancer risk, with each additional serving per day conferring a 20% increase in odds (OR = 1.20, 95% CI 1.02—1.42). Lucenteforte et al.[307] detected a significant positive trend between total energy intake and gastric cancer risk (OR = 1.79, 95% CI 1.16—2.76, highest versus lowest tertile, *p* for trend = 0.01). Sumathi et al.[309] detected a significant inverse association between legume intake and gastric cancer risk (OR = 0.4, 95% CI 0.2—0.9, *p* = 0.05). Finally, Icli et al.[311] concluded that a preference for dried legumes at dinner (OR = 0.7, 95% CI 0.5—1.0, versus no preference, *p* = 0.043) and frequent coffee consumption (OR = 0.3, 95% CI

0.2—0.7, highest versus lowest tertile, *p* for trend <0.0001) were significantly and inversely associated with gastric cancer odds.

With regards to eating habits, Ji et al.[291] demonstrated that increasing temperatures of hot soup or porridge (overall: OR = 1.9, 95% CI 1.5—2.5, burning hot versus not hot, *p* for trend <0.0001; cardia: OR = 2.9, 95% CI 1.7—4.9, burning hot versus not hot, *p* for trend <0.0001; distal: OR = 1.9, 95% CI 1.4—2.5, burning hot versus not hot, *p* for trend <0.0001), frequency of irregular meals (overall: OR = 3.6, 95% CI 2.7—4.9, frequently versus never, *p* for trend <0.0001; cardia: OR = 4.0, 95% CI 2.4—6.5, frequently versus never, *p* for trend <0.0001; distal: OR = 3.7, 95% CI 2.7—5.2, frequently versus never, *p* for trend <0.0001), speed of eating (overall: OR = 2.0, 95% CI 1.8—2.5, fast versus moderate, *p* for trend <0.0001; cardia: OR = 3.8, 95% CI 2.5—5.7, fast versus moderate, *p* for trend <0.0001; distal: OR = 1.8, 95% CI 1.5—2.2, fast versus moderate, *p* for trend <0.0001), and inclination for binge eating (overall: OR = 2.5, 95% CI 2.0—3.1, yes versus no; cardia: OR = 3.0, 95% CI 2.0—4.4, yes versus no; distal: OR = 2.4, 95% CI 1.9—3.1, yes versus no) significantly increased the risks for overall, cardia, and distal gastric cancers; however, no standard definitions were provided for these variables, and the results may be subject to information bias. Ye et al.[293] demonstrated significant positive associations between consumption of moldy foods (OR = 2.32, 95% CI 1.73—3.09, yes versus no, *p* <0.01) and irregular dinners (OR = 5.47, 95% CI 4.22—7.09, at least three times per week versus less than three times per week, *p* <0.01) and the risk of gastric cancer; however, only the association for irregular dinners remained significant upon adjustment for gender, nationality, age, and location of

residence. The authors calculated an adjusted attributable risk of 48.93% of all gastric cancers among the subjects due to irregular dinner patterns (p <0.01). Pourfarzi et al.[308] demonstrated that increasing consumption of hot (OR = 2.85, 95% CI 1.65—4.91, hot versus not hot) and strong tea (OR = 2.64, 95% CI 1.45—4.80, strong versus not strong) significantly increased the odds for gastric cancer in their study population. Compared to dietary components alone, these unhealthy eating behaviors appear to substantially increase gastric cancer risk, but information bias or residual confounding of other diet and lifestyle factors associated with poor eating habits may influence the results.

Table 37 summarizes case-control studies that have evaluated the associations between diet and gastric cancer risk by defining unique dietary patterns among their study populations, largely through the use of principal components analysis. The case-control study by Palli et al.[297] included factor analyses of dietary patterns; increasing consumption of a traditional diet (rich in protein, starch, alcohol, and nitrites) tripled the risk of gastric cancer (OR = 3.0, 95% CI 0.4—0.9, highest versus lowest tertile, p for trend = 0.0001) while a vitamin-rich diet (rich in antioxidant vitamins, fiber, and nitrates) was significantly associated with a 50% reduction in gastric cancer odds (OR = 0.5, 95% CI 0.4—0.7, highest versus lowest tertile, p for trend = 0.0003). In a subsequent analysis from a study cited above, Chen et al.[312] concluded that there were significant differences in distal gastric cancer risk across the six dietary patterns established (p = 0.04) and that high-meat (OR = 2.9, 95% CI 0.89—9.2, p = 0.1) and high-dairy (OR = 2.2, 95% CI 0.68—7.0, p = 0.4) dietary patterns were associated with a non-significant

Table 37. Summary of case-control human studies examining dietary intake patterns and the risk of gastric cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Chen et al.[312]	Population-based case-control Distal gastric cancer cases, n=124 Population controls, n=407	Telephone interview and food frequency questionnaire Six dietary patterns identified via cluster analysis	Overall significant difference in distal gastric cancer risk across dietary patterns (p = 0.04). Non-significant increased risk for distal gastric cancer with high-meat (OR=2.9, 95%CI 0.89—9.2, p = 0.1) and high-milk (OR=2.2, 95%CI 0.68—7.0, p = 0.4) dietary patterns compared to healthy dietary pattern. Intake of red meat significant increased the odds of distal gastric cancer (OR=2.0, 95%CI 0.85—4.7, highest vs. lowest quartile, p for trend = 0.05). Increased intake of fish significantly decreased the risk of esophageal cancer (OR=0.14, 95%CI 0.04—0.48, highest vs. lowest quartile, p for trend = 0.0001).	Also evaluated esophageal cancer (significant inverse trend and quartile ORs for fish intake)
Bahmanyar and Ye[313]	Population-based case-control Gastric cardia cancer cases, n=258 Population controls, n=815	In-person interview and food frequency questionnaire Three dietary patterns identified via principal components analysis	Increasing Western dietary pattern (high in processed meats, red meat, sweets, high-fat dairy, and high-fat gravy) significantly increased odds for gastric cardia cancer (OR=1.8, 95%CI 1.1—2.9, highest vs. lowest tertile of Western diet, p for trend = 0.04). Non-significant decreased odds for gastric cardia cancer with increasing healthy dietary pattern (including fish) (OR=0.7, 95%CI 0.5—1.1, highest vs. lowest tertile of healthy diet, p for trend = 0.13).	Also evaluated esophageal cancer
Bastos et al.[314]	Population-based case-control Gastric cancer cases, n=591 Population controls, n=1463	In-person interview and food frequency questionnaire Serum *H. pylori* status via ELISA Three dietary patterns identified via principal components analysis	Low consumption of dairy, fish and seafood, fruits, salads, vegetables, and meats significantly associated with increased odds of gastric cancer compared to high consumption of dairy, fruits, salads, and vegetables and low consumption of meat and alcohol (OR=1.68, 95%CI 1.31—2.14). This association was similar in cardia (OR=1.71, 95%CI 0.97—3.00) and non-cardia (OR=1.64, 95%CI 1.25—2.14) cancers but stronger in intestinal (OR=1.87, 95% CI 1.30—2.67) versus diffuse (OR=1.32, 95%CI 0.83—2.08) histological type of gastric cancer. *H. pylori* infection was not a significant effect modifier (p = 0.17).	
Bertuccio et	Hospital-based	In-person interview and	Significantly increased risk for gastric cancer with increasing	

Reference	Study Design	Outcome Measurement(s)	Results	Notes
al.[315]	case-control Gastric cancer cases, n=230 Non-cancer controls, n=547	food frequency questionnaire Four dietary patterns identified via principal components analysis	intake of animal products (OR=2.13, 95%CI 1.34—3.40, highest vs. lowest quartile, *p* for trend = 0.0003) or starch-rich (OR=1.67, 95%CI 1.01—2.77, highest vs. lowest quartile, *p* for trend = 0.0463) dietary patterns. Significantly decreased risk for gastric cancer with increasing vitamins and fiber dietary pattern (OR=0.60, 95%CI 0.37—0.99, highest vs. lowest quartile, *p* for trend = 0.0861). Non-significant inverse association between vegetable unsaturated fatty acids pattern and gastric cancer risk (OR=0.89, 95%CI 0.56—1.42, highest vs. lowest quartile, *p* for trend = 0.7325).	

increase in distal gastric cancer risk compared to a healthy diet pattern (comprised mainly of fruits, vegetables, fish, poultry, and dark breads). Bahmanyar and Ye[313] demonstrated that increasing consumption of a Western dietary pattern, high in processed meats, red meat, sweets, high-fat dairy, and high-fat gravy, significantly increased the risk for gastric cardia cancer (OR = 1.8, 95% CI 1.1—2.9, highest versus lowest tertile of Western diet, *p* for trend = 0.04)., while a healthy dietary pattern, which included frequent consumption of fish, non-significantly decreased the risk for gastric cardia cancer (OR = 0.7, 95% CI 0.5—1.1, highest versus lowest tertile of healthy diet, *p* for trend = 0.13). Bastos et al.[314] concluded that a diet pattern with low consumption of dairy, fish and seafood, fruits, salads, vegetables, and meats significantly increased the odds of gastric cancer when compared to a diet pattern that emphasized consumption of dairy, fruits, salads, and vegetables with low meat consumption (OR = 1.68, 95% CI 1.31—2.14). This association was consistent for both cardia (OR = 1.71, 95% CI 0.97—3.00) and non-cardia (OR = 1.64, 95% CI 1.25—2.14) gastric cancers but stronger in intestinal (OR = 1.87, 95% CI 1.30—2.67) versus diffuse (OR = 1.32, 95% CI 0.83—2.08) histological types of gastric cancer. Lastly, Bertuccio et al.[315] concluded that diet patterns with high intakes of animal or starchy foods significantly increased the risk for gastric cancer (OR = 2.13, 95% CI 1.34—3.40, highest versus lowest quartile, *p* for trend = 0.0003), whereas a vitamin- and fiber-rich diet pattern significantly decreased the risk for gastric cancer (OR = 0.60, 95% CI 0.37—0.99, highest versus lowest quartile, *p* for trend = 0.0861). The authors failed to detect a significant association between a diet pattern rich in vegetable unsaturated fatty acids and the risk of gastric cancer (OR = 0.89,

95% CI 0.56—1.42, highest versus lowest quartile, *p* for trend = 0.7325). The evaluation of overall dietary patterns rather than individual dietary components may provide more reliable data regarding the associations between diet and cancer because they are more robust to measurement error and information bias of single components but may result in misclassification bias of individuals that share characteristics of multiple patterns.

A majority of the information regarding diet and gastric cancer risk has been gleaned from case-control studies, which are consistent with respect to negative associations between fruits, fresh vegetables, vitamin C, fiber, and vegetable protein intakes and gastric cancer risk. Associations between fish, fat, and animal or total protein consumption and gastric cancer risk have been less conclusive, but there is some evidence of inverse relationships with vegetable and unsaturated fats and fresh or cooked fish and gastric cancer risk, while preserved or fermented seafood products have been linked to an increased risk for gastric cancer. Additionally, studies that have evaluated total, red, and processed meat consumption have shown positive trends of these foods with gastric cancer risk. Relatively few case-control studies included measurement of *H. pylori* infection status in addition to dietary habits; these studies will be addressed in a subsequent section.

Prospective studies

Compared to retrospective case-control studies, prospective cohort studies provide better evidence of causation for environmental factors such as diet in promoting or reducing cancer risk because of the collection of exposure data prior to any cancer

Table 38. Summary of prospective cohort human studies examining dietary fat, fish, and antioxidant intakes and the risk of gastric cancer.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
Kneller et al.[316]	Prospective cohort of white male life insurance policy holders, n=17,633	Self-administered lifestyle and food frequency questionnaire Death certificates for cause of death and other significant conditions	Cigarette use significantly associated with increased risk of gastric cancer death (HR=2.6, 95%CI 1.1—5.8). Significant increased risk of gastric cancer death with consumption of fresh or frozen fish 1-2 times/month (HR=2.1, 95%CI 1.21—3.55), bacon or side pork 6-13 times/month (HR=2.0, 95% CI 1.02—3.90), greater than 4 cups of milk/day (HR=2.4, 95%CI 1.10—5.04), and apples more than 14 times/month in season (HR=3.2, 95%CI 1.10—9.17). Positive trends between apples, total carbohydrates, and cooked cereals and risk of gastric cancer death were significant.	
Galanis et al.[275]	Population-based prospective cohort of Japanese Hawaiians, n=11,907	Self-administered lifestyle and food frequency questionnaire Cancer incidence via state registry	There was a significant decrease in gastric cancer risk with fruit consumption at least 7 times/week (HR=0.6, 95%CI 0.4—0.9). There was a significant inverse association between fresh fruit and vegetable consumption and gastric cancer risk (8-13 times/week HR=0.5, 95%CI 0.3—0.9; 14+ times/week HR=0.5, 95%CI 0.3—0.8; *p* for trend = 0.02).	FFQ based on 13 foods and 6 beverages over one week.
Ngoan et al.[317]	Prospective cohort study in Fukuoka Prefecture, Japan, n=13,250	Self-administered lifestyle and food frequency questionnaire	High intake of processed meat (HR=3.4, 95%CI 1.4—8.1) and pickled food (HR=2.6, 95%CI 1.1—5.8) significantly increased gastric cancer incidence risk in men only. Medium (HR=2.2, 95%CI 1.2—5.2) and high (HR=2.7, 95%CI 1.2—6.1) use of cooking oil significantly increased risk of gastric cancer incidence in men only (*p* for trend <0.05). High intake of liver significantly increased gastric cancer incidence risk in women only (HR=2.9, 95%CI 1.1—7.5, significant trend). High intake of suimono soup significantly increased risk of gastric cancer incidence in both sexes (for men HR=2.4, 95%CI 1.0—5.8, *p* for trend <0.05; for women HR=4.1, 95%CI 1.2—14.2, *p* for trend <0.05). Positive trends for processed meats and cooking oil significant when excluding the first 3 years of follow-up (*p*	Authors did not indicate how gastric cancer incidence and deaths were ascertained.

Reference	Study Design	Outcome Measurement(s)	Results	Notes
			for trend <0.05). Inverse trend for green and yellow vegetables significant when excluding the first 3 years of follow-up (*p* for trend <0.05).	
Tsugane et al.[318]	Population-based prospective cohort of Japanese adults, n=39,065	Self-administered lifestyle and food frequency questionnaire Gastric cancer incidence via national registry Mortality data via death certificates	There was a significant positive association between salt intake and gastric cancer risk in men only (*p* for trend <0.001). There was a borderline significant positive association between miso soup consumption and gastric cancer risk in men only (HR=1.40, 95%CI 0.97—2.03, 3+ cups/day versus not daily). Significant positive trends for salted fish roe (HR=2.21, 95%CI 1.24—3.92, almost everyday versus almost none, adjusted for demographics and salt intake) and salted fish preserves (HR=2.76, 95%CI 1.44—5.27, almost everyday versus almost none, adjusted for demographics and salt intake) were also detected. Significant positive trends for pickled vegetables and dried or salted fish were attenuated after adjustment for salt intake and residential area. In women, there was a significant positive trend for salted fish roe and gastric cancer risk (HR=3.37, 95%CI 1.48—7.66, almost everyday versus almost none, adjusted for demographics and salt intake) and a borderline significant positive trend for pickled vegetables intake and gastric cancer risk (HR=2.01, 95%CI 0.97—4.17, almost everyday versus almost none, adjusted for demographics and salt intake). These trends were attenuated following adjustment for salt intake and residential area.	
Lee et al.[319]	Population-based prospective cohort of women in Iowa, n=34,708	Self-administered lifestyle and food frequency questionnaire Gastric cancer incidence via state registry	There was a significant inverse association between dietary zinc and gastric or esophageal cancer risk (HR=0.13, 05%CI 0.03—0.63, highest versus lowest intake, *p* for trend <0.01). There was a borderline positive trend between dietary heme iron and gastric or esophageal cancer risk (HR=2.83, 95%CI 0.84—9.54, highest versus lowest intake, *p* for trend = 0.06).	Also evaluated esophageal cancer risk
Larsson et al.[320]	Population-based prospective	Self-administered lifestyle and food frequency questionnaire	There was a significant positive association between processed meat consumption and gastric cancer risk	Second questionnaire sent to all participants 7

Reference	Study Design	Outcome Measurement(s)	Results	Notes
	cohort of Swedish women, n=61,433	Gastric cancer incidence and mortality data via national registries	(HR=1.66, 95%CI 1.13—2.45, highest versus lowest intake, *p* for trend = 0.01). There were no significant trends for fish intake. There was a significant positive association between NDMA intake and gastric cancer risk (HR=1.96, 95%CI 1.08—3.58, highest versus lowest intake, *p* for trend = 0.02). There was a borderline significant interaction between NDMA and fruit and vegetable consumption with regards to gastric cancer risk (*p* = 0.06).	years following end of recruitment
Takachi et al.[321]	Population-based prospective cohort of Japanese adults, n=77,500	Self-administered lifestyle and food frequency questionnaire Gastric cancer incidence and mortality data via patient notification and national registries	There were significant positive trends for pickled vegetables (HR=2.24, 95%CI 1.71—2.93, highest versus lowest intake, *p* for trend <0.01) and salted fish roe (HR=1.66, 95%CI 1.32—2.09, highest versus lowest intake, *p* for trend <0.01) and gastric cancer risk. Dried and salted fish intake in the top 4 quintiles significantly increased the risk for gastric cancer, but the linear trend was not significant (HR=1.46, 95%CI 1.14—1.88, highest versus lowest intake, *p* for trend = 0.12).	Second questionnaire sent to all participants 5 years following end of recruitment Also evaluated other cancers and cardiovascular diseases

Abbreviation:
NDMA = *N*-nitrosodimethylamine

diagnosis; however, these studies are subject to limitations such as loss to follow-up or measurement error. Given the much larger sample sizes of prospective cohort studies, most employ self-administered measures of dietary intake, such as FFQs, which may lead to information biases due to inaccurate or incomplete reporting on the part of the subject or substantial changes in dietary habits during the years of follow-up.

Several prospective cohort studies have examined fish, fat, and antioxidant intake with respect to gastric cancer risk and are summarized in Table 38. Kneller et al.[316] established a cohort of white male life insurance customers in the United States; after 20 years of follow-up, consumption of fresh or frozen fish once or twice monthly was associated with a significantly increased risk of death due to gastric cancer (hazard ratio (HR) = 2.2, 95% CI 1.21—3.55). High intakes of bacon or side pork (HR = 2.0, 95% CI 1.02—3.90, six to thirteen times per month versus less than three times per month), milk (HR = 2.4, 95% CI 1.10—5.04, greater than four cups per day versus less than one cup per day), and apples (HR = 3.2, 95% CI 1.10—9.17, more than fourteen servings versus less than one serving per month in season) were also associated with significantly increased risks for gastric cancer death.

In a prospective cohort study of men and women of Japanese descent residing in Hawaii, Galanis et al.[275] noted a significant decrease in gastric cancer risk with consumption of fresh fruit at least seven times per week (HR = 0.6, 95% CI 0.4—0.9). A similar significant trend was seen when high intakes (at least eight times per week) of fresh fruit and vegetables were compared to intakes of seven or fewer times per week. However, a drawback of this study was the assessment of dietary intakes with an FFQ

consisting of only thirteen foods and six beverages over one week; such measurement may not accurately reflect typical dietary intake over long periods of time.

Ngoan et al.[317] conducted a large prospective cohort study in the Fukuoka Prefecture in Japan. Among men, consuming pickled foods at least twice daily conferred a significantly increased risk of developing gastric cancer (HR = 2.6, 95% CI 1.1—5.8, versus two to four servings per week), while consuming processed meat at least once daily more than tripled gastric cancer risk (HR = 3.4, 95% CI 1.4—8.1, versus four or fewer servings per month). The use of any cooking oil at least twice weekly also significantly increased the risk of gastric cancer in a dose-dependent manner (for use two to four times weekly: HR = 2.2, 95% CI 1.2—5.2; for daily use: HR = 2.7, 95% CI 1.2—6.1; p for trend <0.05, versus four or fewer servings per month). Among women, consuming liver at least twice weekly significantly increased the risk for gastric cancer (HR = 2.9, 95% CI 1.1—7.5, versus seldom or never). Within both sexes, consumption of suimono soup (a clear soup made from pig stomach or intestines and mushrooms[322]) at least daily significantly increased the risk for gastric cancer (for men HR = 2.4, 95% CI 1.0—5.8; for women HR = 4.1, 95% CI 1.2—14.2, versus four or fewer servings per month). Additional analyses excluding the first three years of follow-up demonstrated significant positive trends for processed meat and cooking oil and significant negative trends for green and yellow vegetables among the overall cohort (p <0.05); exclusion of the first few years of follow-up would eliminate individuals with pre-clinical gastric cancer at baseline who may have changed their dietary patterns compared to earlier in their lives.

In another prospective cohort study of Japanese adults, Tsugane et al.[318] demonstrated significant positive trends for salted fish roe (HR = 2.21, 95% CI 1.24—3.92, almost every day versus almost none, adjusted for demographics and salt intake) and salted fish preserves (HR = 2.76, 95% CI 1.44—5.27, almost every day versus almost none, adjusted for demographics and salt intake) and gastric cancer incidence among men only. There was a borderline significant positive trend between miso soup intake and gastric cancer risk (HR = 1.40, 95% CI 0.97—2.03, at least three cups daily versus less than daily). Among female cohort members, increasing intake of salted fish roe was associated with a significant increase in gastric cancer risk (HR = 3.37, 95% CI 1.48—7.66, almost every day versus almost none, adjusted for demographics and salt intake), while the positive association between pickled vegetables and gastric cancer risk was borderline significant (HR = 2.01, 95% CI 0.97—4.17, almost every day versus almost none, adjusted for demographics and salt intake). Many of these associations were attenuated upon adjustment for residential area but suggested that high intakes of salted foods increase the risk of gastric cancer in both men and women.

Although antioxidant function or consumption were not directly addressed, Lee et al.[319] measured a significant inverse association between dietary zinc (a component of many enzymes, including SOD) and gastric and esophageal cancer risk among women in the Iowa Women's Health Study (HR = 0.13, 95% CI 0.03—0.93, highest versus lowest intake, p for trend <0.01). Conversely, heme iron (a component of catalase) was positively associated with gastric and esophageal cancer risk (HR = 2.83, 95% CI 0.84—9.54, highest versus lowest intake, p for trend = 0.06). Additional analyses showed that

the inverse association for dietary zinc remained significant for gastric cancer incidence alone ($p = 0.03$); however, the positive association between heme iron and gastric cancer was not significant ($p = 0.18$).

In a subsequent prospective cohort of Swedish women, Larsson et al.[320] documented a significant positive association between the consumption of processed meat and the risk of gastric cancer (HR = 1.66, 95% CI 1.13—2.45, highest versus lowest tertile, p for trend = 0.01). A specific marker of nitrosamines (carcinogenic compounds) frequently found in processed meats, *N*-nitrosodimethylamine (NDMA), was also significantly associated with an increased risk of gastric cancer (HR = 1.96, 95% CI 1.08—3.58, highest versus lowest quintile, p for trend = 0.02). When NDMA exposure was evaluated in conjunction with fruit and vegetable intake due to the latter's putative preventive effects, there was a borderline significant interaction between fruits and vegetables and NDMA (p for trend = 0.06).

Finally, a Japanese cohort study conducted by Takachi et al.[321] evaluated the risks of multiple cancers and cardiovascular disease with respect to frequent consumption of salt and salted foods. In this population, the highest intake of salted vegetables imparted more than doubled the risk of developing gastric cancer (HR = 2.24, 95% CI 1.71—2.93, highest versus lowest quintile, p for trend <0.01), while frequent intake of salted fish roe increased the risk of gastric cancer by 66% (HR = 1.66, 95% CI 1.32—2.09, highest versus lowest quintile, p for trend <0.01). Frequent intake of dried and salted fish was also associated with a significant increase in gastric cancer risk, although

the linear trend among the quintiles was not significant (HR = 1.46, 95% CI 1.14—1.88, highest versus lowest quintile, *p* for trend = 0.12).

In summation, although limited in scope and country of origin, prospective cohort studies that have evaluated fish, fat, and antioxidant consumption and the risk of gastric cancer have shown positive associations between salted fish products, preserved meats, and salted vegetables and gastric cancer risk as well as negative associations with regards to fruit and vegetable consumption. Additionally, one study showed a positive association with fresh or frozen fish consumption, and another study concluded that the use of cooking oil increased the risk of gastric cancer; however, both of these results were demonstrated in men only. Prospective cohort studies offer a number of strengths compared to case-control studies but are more costly and time-consuming, thereby resulting in fewer studies from which to draw conclusions. The associations between salted or processed fish, vegetables, or meat and increased gastric cancer risk shown by many case-control studies were borne out by the prospective cohort studies conducted, but there are too few data to conclude that frequent consumption of fresh or frozen fish or vegetable oils increase or decrease the risk of gastric cancer.

Intervention trials

Human clinical intervention trials for antioxidant intake and gastric cancer risk are limited; there have been no studies examining interventions of dietary fish or fat and gastric cancer incidence or risk biomarkers. Table 39 describes relevant human intervention studies of dietary or anti-inflammatory treatments and gastric cancer risk and

Table 39. Summary of human intervention studies examining dietary intake patterns and the risk of gastric cancer.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
Takahashi et al.[323]	Healthy volunteers, 40-69 y, n=550	1-year crossover: • Education to decrease sodium intake and increase carotene and vitamin C intake during the first year • Education to decrease sodium intake and increase carotene and vitamin C intake during the second year	Dietary energy, sodium, carotene, and vitamin via self-administered food frequency questionnaire Urinary sodium Serum alpha- and beta-carotene Serum ascorbic acid	Significant 384 mg/d decrease in sodium intake in intervention group versus 255 mg/d increase in control group (p <0.001). Significant 418 µg/d increase in carotene intake in intervention group versus 220 µg/d increase in control group (p <0.05). Significant 13 mg/d increase in vitamin C intake in intervention group versus 2 mg/d increase in control group (p <0.05). These changes corresponded to changes in consumption of salted foods and green and yellow vegetables. There were significant differences in the changes of urinary sodium excretion in the intervention versus control groups (p <0.001). Only serum alpha-carotene differed significantly between the intervention and control groups (p <0.01).	Although study objectives mentioned gastric cancer, outcomes assessed for intervention effectiveness not gastric cancer risk reduction.
Hao et al.[324]	Individuals with medical history of stomach disorder, family history of cancer, or smoking and/or alcohol use, 34-74 y, n=5033	Double-blind 2-year intervention with 10 y follow-up: • 2 200 mg synthetic allitridum daily and 100 µg selenium every other day for one month during each year • Placebo capsules of corn oil and starch in the same pattern	Gastric cancer incidence via village doctor monitoring Cause of death from death certificates Assessed for first 5 y of follow-up	Gastric cancer incidence declined by 47.3% in the intervention compared to control group (not stated if statistically significant). There was a significant reduction in gastric cancer incidence in men only in the intervention group (OR=0.36, 95%CI 0.14—0.92), after controlling for age, family history, smoking, alcohol, and history of stomach illness.	Allitridum comprises almost 45% of garlic oil. Too few person-years among females to determine effect of intervention.
You et al.[325]	Randomly selected healthy	Randomized double-blind factorial design following stratification by *H. pylori*	*H. pylori* via ELISA Gastroscopy including biopsy	For *H. pylori* treatment group, histological distributions different significantly from placebo group (p = 0.009 for 1999 and p =	

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
	individuals, 35-64 y, n=3365	infection status: • Placebo twice daily for 2 w • 1 g amoxicillin and 20 mg omeprazole twice daily for 2 w • 250 mg vitamin C, 100 IU vitamin E, and 37.5 µg selenium twice daily for 7.3 y • Placebo twice daily for 7.3 y • 200 mg aged garlic extract and 1 mg steam-distilled garlic oil twice daily for 7.3 y • Placebo twice daily for 7.3 y • All possible combinations of above	and histopathological scoring of gastritis, metaplasia, dysplasia, and cancer Gastric cancer diagnosis via medical records	0.0001 for 2003 follow-ups). Treatment groups had higher mild chronic and superficial gastritis and less deep intestinal metaplasia than placebo group. *H. pylori* treatment significant decreased the odds of an advanced gastric lesion (OR=0.77, 95%CI 0.62—0.95 in 1995; OR=0.60, 95%CI 0.47—0.75 in 2003). There were no significant effects of vitamin or garlic interventions on gastric cancer incidence or gastric histopathological distribution. There were no significant differences in mortality between any of the groups.	
Tu et al.[326]	Randomly selected healthy individuals, 35-64 y, n=3355	Sub-analysis by *MnSOD* genotype of above study Randomized double-blind factorial design following stratification by *H. pylori* infection status: • Placebo twice daily for 2 w • 1 g amoxicillin and 20 mg omeprazole twice daily for 2 w	*H. pylori* via ELISA Gastroscopy including biopsy and histopathological scoring of gastritis, metaplasia, dysplasia, and cancer *MnSOD* genotyping by PCR	There was a weak positive association between the variant *MnSOD* alleles and dysplasia risk (OR=1.31, 95%CI 1.02—1.68), after controlling for age, sex, *H. pylori*, smoking, and drinking. The risk from the variant alleles was significantly higher among *H. pylori*-positive versus *H. pylori*-negative subjects (OR=4.01, 95%CI 2.80—5.75 for dysplasia). Among smokers, the risk from the variant alleles was significantly increased	Variant *MnSOD* predicted to decrease MnSOD transport into mitochondria, therefore increasing ROS exposure.

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
		• 250 mg vitamin C, 100 IU vitamin E, and 37.5 µg selenium twice daily for 7.3 y • Placebo twice daily for 7.3 y • 200 mg aged garlic extract and 1 mg steam-distilled garlic oil twice daily for 7.3 y • Placebo twice daily for 7.3 y • All possible combinations of above		compared to non-smokers (OR=1.96, 95%CI 1.27—3.03). Among individuals receiving the vitamin intervention, *H. pylori*-positive subjects with the variant alleles had significantly increased gastric pathological regression (OR=2.45, 95%CI 1.37—4.38) compared to the wild-type allele.	
Ma et al.[327]	Randomly selected healthy individuals, 35-64 y, n=3365	14.7 y follow-up of above study Randomized double-blind factorial design following stratification by *H. pylori* infection status: • Placebo twice daily for 2 w • 1 g amoxicillin and 20 mg omeprazole twice daily for 2 w • 250 mg vitamin C, 100 IU vitamin E, and 37.5 µg selenium twice daily for 7.3 y • Placebo twice daily for 7.3 y • 200 mg aged garlic	*H. pylori* via ELISA Gastroscopy including biopsy and histopathological scoring of gastritis, metaplasia, dysplasia, and cancer Gastric cancer diagnosis via medical records	There was a significant decrease in gastric cancer incidence among subjects receiving *H. pylori* treatment compared to the placebo group (OR=0.61, 95%CI 0.38—0.96). The vitamin treatment group experienced significantly fewer deaths from gastric or esophageal cancer than the placebo group (HR=0.51, 95%CI 0.30—0.87). This association was borderline significant when looking at gastric cancer deaths only (p = 0.06).	

Reference	Subjects	Intervention	Outcome Measurement(s)	Results	Notes
		extract and 1 mg steam-distilled garlic oil twice daily for 7.3 y • Placebo twice daily for 7.3 y All possible combinations of above			
Wong et al.[328]	Randomly selected *H. pylori*-positive subjects with atrophic gastritis, metaplasia, or dysplasia from above study, 35-64 y, n=1024	Randomized double-blind factorial intervention: • *H. pylori* placebo for 1 w followed by celecoxib placebo for 24 mos • 20 mg omeprazole, 1 g amoxicillin, 500 mg clarithromycin twice daily for 1 w followed by 200 mg celecoxib twice daily for 24 mos • 20 mg omeprazole, 1 g amoxicillin, 500 mg clarithromycin twice daily for 1 w followed by 200 mg celecoxib twice daily for 24 mos celecoxib placebo for 24 mos • Placebo of *H. pylori* treatment for 1 w followed by celecoxib placebo for 24 mos	^{13}C-UBT breath test for *H. pylori* Gastroscopy including biopsy and histopathological scoring of gastritis, metaplasia, dysplasia, and cancer Gastric cancer diagnosis via medical records	Celecoxib (OR=1.55, 95%CI 1.01—2.38) and *H. pylori* (OR=1.80, 95%CI 1.16—2.78) treatments significantly increased regression compared to placebos. Effect of two treatments together was not significant. 7 of 9 gastric cancer cases were diagnosed during treatment, so cancer incidence was not evaluated as an endpoint.	COX-2 overexpression seen in *H. pylori*-associated gastric cancers.

biomarkers. Each of these studies was conducted in the high-risk countries of Japan or China.

Takahashi et al.[323] established a one-year crossover intervention trial involving education on a diet low in sodium and high in vitamin C and carotenoids in an effort to reduce the risk of gastric cancer and cardiovascular disease in this high-risk population. Although the intervention appeared effective—subjects receiving the intervention significantly reduced sodium intake and increased vitamin C and carotene intake—data have not been published so far quantifying the effect of the intervention on gastric cancer risk[329, 330]. It is not clear if the investigators intend to collect such data.

In a large clinical trial of individuals with a history of gastric disorders, family history of cancer, or use of tobacco and/or alcohol, Hao et al.[324] tested the effects of allitridum (a component of garlic oil) and selenium on gastric cancer incidence and mortality. The treatment continued for two years, and subjects were continuously followed for an additional ten years. After five years of follow-up, gastric cancer incidence decreased by 47.3% in the treatment group compared to the control group; it was not reported if this difference was statistically significant. In logistic regression models that controlled for multiple confounding factors, a significant reduction in gastric cancer risk with allitridum and selenium treatment was evident only among men in the study (OR = 0.36, 95% CI 0.14—0.92). This association was not seen among the women in the study possibly due to low numbers of gastric cancer cases among the female subjects.

You et al.[325] first reported a population-based randomized double-blind clinical trial involving several treatments (anti-*H. pylori* medication; vitamin C, vitamin E, and selenium; aged garlic extract and garlic oil) and combinations thereof. Subjects were monitored via gastric biopsy for histological changes predictive of cancer as well as frank gastric cancer diagnosis via active medical follow-up. Anti-*H. pylori* treatment significantly reduced the risk of an advanced gastric lesion compared to placebo (OR = 0.60, 95% CI 0.47—0.75 at the 2003 follow-up). However, there were no significant effects of the vitamin/mineral or garlic supplements on gastric histopathology or cancer incidence. All further intervention studies discussed are additional analyses of this parent study.

To assess the effects of genetic polymorphisms of an enzyme involved in the clearance of ROS, *MnSOD*, Tu et al.[326] concluded that variant forms of this enzyme with impaired effectiveness significantly increased the risk of gastric dysplasia, especially among individuals positive for *H. pylori* (OR = 4.01, 95% CI 2.80—5.75). A significant increase in risk was also observed among smokers with the variant allele compared to smokers with the wild-type allele (OR = 1.96, 95% CI 1.27—3.03). When comparing the treatments in concert with *MnSOD* genotype, the authors showed that *H. pylori*-positive, variant *MnSOD* subjects receiving the vitamin/mineral supplement showed an increased rate of gastric pathological regression compared to those with the wild type allele (OR = 2.45, 95% CI 1.37—4.38). This result suggests that the vitamin/mineral supplement was able to counteract the pro-oxidant effects of *H. pylori*

infection in the presence reduced MnSOD activity, thereby delaying the progression of mild gastric conditions to pre-cancerous lesions.

After nearly 15 years of follow-up, Ma et al.[327] confirmed that the subjects receiving the *H. pylori* treatment had significantly decreased risk of gastric cancer compared to subjects receiving the placebo (OR = 0.61, 95% CI 0.38—0.96). However, no significant differences in gastric cancer incidence were observed in the vitamin/mineral or garlic supplement groups. When gastric and esophageal cancer deaths were evaluated together, the vitamin/mineral supplement significantly decreased mortality compared to the placebo group (HR = 0.51, 95% CI 0.30—0.87). This association was borderline significant for gastric cancer death alone as an endpoint (HR = 0.55, 95% CI 0.29—1.03, $p = 0.06$).

Lastly, Wong et al.[328] conducted an additional intervention in those subjects that were positive for *H. pylori* infection and atrophic gastritis, metaplasia, or dysplasia from the gastric biopsy. These subjects were randomly assigned to a one-week *H. pylori* treatment with or without subsequent two-year celecoxib (a COX-2 inhibitor) treatment or respective placebos. Either treatment on its own significantly increased the regression of gastric histopathology relative to its treatments (celecoxib: OR = 1.55, 95% CI 1.01—2.38; *H. pylori* treatment: OR = 1.80, 95% CI 1.16—2.78); however, the effect of both treatments together was not significant when compared to treatment with a placebo. This suggests that the treatments share a common mechanism of decreasing inflammation, and both treatments together do not confer any additional benefit. Because a majority of the

gastric cancers diagnosed among the study subjects occurred during the treatment period, gastric cancer incidence was not assessed as an outcome.

From the available clinical data, it appears unlikely that garlic and antioxidant nutrient supplementation decreases the risk of gastric cancer. However, each study utilized oral supplements of purified components, such as ascorbic acid or allitridum. It is not clear what effects elevated intakes of vitamins C and/or E, selenium, and garlic as part of an overall healthy diet low in fat, processed foods, and red meat would have on gastric cancer risk or gastric histopathology. Although no intervention trial directly evaluated dietary PUFA or fish intake on gastric cancer incidence, the most recent study by Wong et al.[328] implicated COX-2 inhibition in promoting regression of pre-cancerous gastric conditions. This finding may apply to n-3 PUFA such as DHA and ALA, which have been shown to inhibit COX-2[3, 148, 331]. More clinical intervention research with human subjects is needed on dietary PUFA and fish intake and gastric cancer incidence.

Interaction of dietary factors with H. pylori *infection*

As mentioned previously, a majority of population-based studies of dietary intakes and gastric cancer risk have not included evaluation of *H. pylori* infection status; this is likely as result of the expense of such tests as well as their low sensitivity and specificity in cases of chronic gastritis[332]. There is some evidence from the studies presented in Table 36 demonstrating an interaction between *H. pylori* infection and

certain dietary components, such as sodium and antioxidants, in increasing or decreasing the odds of gastric cancer.

Lee et al.[300] concluded that *H. pylori*-positive individuals with a taste preference for salty foods (>0.3% NaCl) had ten times the risk of gastric cancer compared to *H. pylori*-negative subjects with a low salt preference (OR = 10.1, 95% CI 3.4—30.0). Among *H. pylori*-positive subjects with a preference for less salty foods (≤0.3% NaCl), there was an insignificant increase in gastric cancer risk (OR = 1.7, 95% CI 0.6—4.7). Because the number of subjects in this study was relatively small (n=69 cases), the confidence intervals are wide, and the results may not apply to the general population. Nevertheless, the authors determined that the interaction between *H. pylori* infection and preference of salt was statistically significant in their study population (p = 0.047). Subsequently, a larger hospital-based case-control study (n=329 cases) conducted by Phukan et al.[302] also showed significant associations between *H. pylori* infection and the salt-rich foods smoked salted meat (OR = 1.9, p <0.046) and sa-um (fermented pork fat, OR = 2.1, p <0.02).

A hospital-based case-control study conducted by Kim et al.[301] showed significant protective effects of vitamins E (OR = 0.16, 95% CI 0.03—0.83, highest versus lowest tertile, p for trend = 0.028) and C (OR = 0.10, 95% CI 0.02—0.63, highest versus lowest tertile, p for trend = 0.015) among *H. pylori*-positive subjects; however, the interactions of these antioxidants with *H. pylori* infection were not statistically significant. The results from Pourfarzi et al.[308] and Pakseresht et al.[310] previously discussed included adjustment for *H. pylori* status, suggesting that the protective effects

of allium vegetables, fruits, fish, protein, vitamin C, zinc, and iron and risk-enhancing effects of red meat, dairy products, and fat observed in these studies are independent of *H. pylori* infection. Additionally, the population-based case-control study conducted by Wu et al.[304] mentioned that exploratory analyses of the 33-50% of subjects with *H. pylori* results did not affect the positive association with total fat or inverse association with dietary fiber. Because *H. pylori* infection is considered a greater risk factor for non-cardia than cardia gastric cancers, it is not surprising that dietary factors appear to alter the risk of gastric cancers irrespective of *H. pylori* infection status, especially those cancers affecting the gastric cardia, as in Wu et al.[304].

Conclusions

Although gastric cancer remains one of the deadliest cancers worldwide, data regarding the role of diet as a risk modifier of gastric cancer are relatively limited. There are few *in vitro* and animal model studies examining the associations between diet and gastric cancer onset and progression. Case-control studies have provided some evidence that plant fats and PUFA reduce the risk while saturated fatty acids increase the risk of gastric cancer. There is also evidence that increased consumption of fresh fruits and vegetables may decrease the risk of gastric cancer, possibly due to antioxidants and/or dietary fiber present within the plants. Frequent consumption of salted or fermented fish, meat, or vegetables likely increases the risk of gastric cancer; however, it has not been demonstrated conclusively that fresh fish intake increases or decreases the risk of gastric cancer.

Data from the numerous case-control studies have been somewhat supported by the small number of prospective cohort studies. Consumption of pickled or salted foods (including fish) imparted significantly increased risks for gastric cancers in Swedish and Japanese populations, but the prospective studies did not yield sufficient data regarding fresh fish or dietary fat intakes and the risk of gastric cancer. These studies were also limited regarding their assessment of antioxidant exposure or status; future prospective analyses would benefit from the use of objective measures of antioxidant status to examine the association of multiple antioxidants (both endogenous and exogenous) with gastric cancer risk.

While there have been several clinical trials involving antioxidant supplementation and gastric cancer risk, most have shown that supplementation was not adequate to reduce the risk of cancer in individuals positive for *H. pylori* infection. A notable exception was individuals with the variant *MnSOD* gene, who benefited from the vitamin and mineral supplement[326]. All intervention studies were conducted in the high-risk countries of Japan and China, which have very high rates of *H. pylori* prevalence. While a high-risk population is often necessary for dietary intervention studies, data gleaned from such studies are generally not applicable to a healthy population. The presence of *H. pylori* infection adds an additional limitation, as it is a major risk factor for non-cardia—but not cardia—gastric cancer. Cardia cancer may be more susceptible to risk reduction via dietary intervention, but its prevalence is only beginning to increase. Future research regarding gastric cardia cancer risk reduction will

require further evaluation of diet and this specific cancer site, especially through laboratory-based and prospective cohort studies.

Unfortunately, *H. pylori* infection remains a substantial burden in many parts of the world. There are promising data suggesting a protective effect of dietary antioxidant intake among *H. pylori*-positive individuals regarding gastric cancer development, and high intakes of salt and salted foods may act synergistically with *H. pylori* infection to increase the risk of gastric cancer. More research is needed to ascertain if the anti-inflammatory effects of n-3 attenuate the risk of gastric cancer among *H. pylori*-positive individuals. The relationships between dietary fish, fat, and antioxidants and the risk of gastric cancer remain inconclusive and poorly characterized and would benefit from additional prospective analyses.

Chapter VII: Dietary fish, fat, and antioxidant consumption and the risk of gastric cancer in the Singapore Chinese Health Study

Introduction

Gastric cancer is the third leading cause of cancer death in men and fifth leading cause of cancer death in women worldwide[264]. Infection with *Helicobacter pylori* (*H. pylori*) is an established risk factor for non-cardia gastric cancer, the incidence of which has decreased over the past three decades, likely due to increased sanitation and access to refrigeration and antibiotic treatment[266, 272-274]. However, the incidence of cardia gastric cancer has been increasing in wealthy countries, and dietary factors such as fat, fiber, red meat, fruits, and high-fat dairy have been shown to modify the risk of cardia gastric cancer[266, 304, 306, 313, 333]. In Singapore, it has been recently reported that 16% of all gastric cancers affect the cardia, double the proportion reported from 1993-1997[267].

Of particular interest regarding nutritional risk factors for gastric cancer is the intake of fish and dietary fat. Omega-3 fatty acids (n-3), such as those found in fish as well as nuts and seeds, have been shown to reduce the risk of breast and prostate cancers, possibly due to decreased inflammatory responses, modifications of signal transduction pathways, and stimulation of tumor cell apoptosis by lipid peroxidation at gastric pH[2, 6, 7, 276, 334]. On the other hand, lipid peroxidation produces reactive metabolites that may modify DNA, leading to point mutations of the tumor suppressor *p53*[276, 335, 336]. As one of the first tissues exposed to lipid peroxidation metabolites following dietary consumption of n-3, an examination of modulation of gastric cancer risk by dietary fish and fat intake would provide additional information on the roles of n-3 intake and subsequent lipid peroxidation in the risk of human cancers.

A recent meta-analysis by Wu et al.[277] concluded that high fish consumption reduced the risk of gastric cancer by thirteen percent (OR = 0.87, 95% CI = 0.71 – 1.07) based upon data from 15 case-control and two prospective cohort studies. Because of the limited data from prospective cohort studies, an analysis of the Singapore Chinese Health Study is warranted. In this study of more than 60,000 individuals, detailed dietary information was collected at baseline prior to the diagnosis of any cancer, and participants were followed for an average of twelve years[278, 279]. If significant associations are detected in the overall cohort, the collection of biospecimens from approximately 60% of participants would allow for *H. pylori* testing to control for this important risk factor, which may be present in 43% of Singapore Chinese individuals[278, 281]. We hypothesize that fish and n-3 intakes are inversely associated with gastric cancer risk in the Singapore Chinese Health Study.

Materials and Methods

Singapore Chinese Health Study

The design and inclusionary and exclusionary criteria for the Singapore Chinese Health Study have been previously described[280]. Cohort members were selected from permanent residents or citizens of Singapore residing in government housing and between the ages of 45 and 74; all cohort members belonged to one of the two major Chinese dialect groups (Cantonese and Hokkien)[279]. At baseline, all cohort members completed an in-person interview with a trained study staff member that included a validated 165-item food frequency questionnaire (FFQ). In conjunction with the FFQ

validation, the Singapore Food Composition Database was developed using food composition data from China, Malaysia, Taiwan, and Hawaii; from this database, mean daily consumption values for 96 dietary components including fats and micronutrients were ascertained for each cohort member[279]. The Singapore Chinese Health Study was approved by the Institutional Review Boards at the National University of Singapore and the University of Pittsburgh.

Cancer diagnoses and deaths from cancer were identified by linking cohort members to the Singapore Cancer Registry and the Singapore Registry of Births and Deaths. This comprehensive national registry was established in 1968, and as of April 2008, only 27 cases were lost to follow-up due to migration out of Singapore. We used data from the 27,293 men and 34,028 women who did not have a history of cancer diagnosis at baseline, based on self-report and computer-assisted record linkage analysis with the Singapore Cancer Registry. As of December 31, 2008 (an average follow-up of 12.3 years), 519 of these cohort members had been diagnosed with gastric cancer (ICD-10 16.0). Of these cases, 73 affected the gastric cardia (ICD-10 C16.0), 345 affected the gastric non-cardia (ICD-10 C16.1 – C16.8), and 101 were unspecified (ICD-10 C16.9).

Cox proportional hazards regression analysis of diet, environmental factors, and gastric cancer incidence

Person-years of follow-up were computed from the recruitment date to the date of gastric cancer diagnosis, death, migration, or December 31, 2008, whichever occurred first. A series of Cox proportional hazards regression models were performed to test the

associations between dietary fish, fat, and antioxidant intakes and gastric cancer incidence. For dietary fish, fat, and micronutrient antioxidants, exposures were quantified from sex-specific nutrient density (food or nutrient weight relative to daily energy intake) quartiles based upon the distribution across the entire cohort. For tea and coffee consumption, the lowest quartile consisted of never drinkers, and the top three quartiles were based upon the distribution among ever drinkers. A complete list of all exposures evaluated is provided in Table 40. To control for confounding, the following were included as covariates: sex (male/female), dialect group (Cantonese/Hokkien), age (years), interview year (1993-1995/1996-1998), education (less than secondary/secondary or higher), tobacco smoking (ever/never), alcohol use (ever/never), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and daily energy intake (kcal/d).

Table 40. Fish, fat, and antioxidant variables evaluated via Cox proportional hazards regression.

Primary Exposure Variables (food or nutrient density quartiles)	**Antioxidant Exposure Variables (nutrient density quartiles)**
All fish and shellfish (g/kcal)	Selenium (μg/kcal)
Fresh fish and shellfish (g/kcal)	Total carotenoids (μg/kcal)
Preserved fish and shellfish (g/kcal)	α-carotene (μg/kcal)
Fish, fresh and preserved (g/kcal)	β-carotene (μg/kcal)
Shellfish, fresh and preserved (g/kcal)	β-cryptoxanthin (μg/kcal)
Total fat (g/kcal)	Lycopene (μg/kcal)
Saturated fat (g/kcal)	Lutein (μg/kcal)
Monounsaturated fat (g/kcal)	Total vitamin C (mg/kcal)
PUFA (g/kcal)	Total vitamin E (mg α-tocopherol equivalents/kcal)
n-3 fat (g/kcal)	Total soy isoflavones (mg/kcal)
Marine n-3 (g/kcal)	Genistein (mg/kcal)
Non-marine n-3 (g/kcal)	Daidzein (mg/kcal)
n-6 fat (g/kcal)	Glycitein (mg/kcal)
Animal fat (g/kcal)	Total isothiocyanate (μmol/kcal)
Red meat fat (g/kcal)	Black tea (cups/month)
Plant fat (g/kcal)	Green tea (cups/month)
Cholesterol (mg/kcal)	Any tea (cups/month)
	Coffee (cups/week)

Statistical analysis

SAS v9.2 (SAS Institute, Inc., Cary, NC) was used for statistical analyses. Statistical analyses were conducted for the overall cohort, within men, and within women for all gastric cancers, cardia gastric cancers, non-cardia gastric cancers, and unspecified gastric cancers. All hazard ratios (HRs) and 95% confidence intervals (CIs) reported were two-sided and computed relative to the lowest quartile or category of intake, and $\alpha = 0.05$ was set as the cutoff for determining statistical significance. The linear tests for exposure-gastric cancer trends were computed based on ordinal values for the categories (0,1,2,3, etc.).

Results

The distributions of demographic characteristics of cohort members within each quartile of total fat intake and stratified by sex are shown in Table 41. Individuals in the highest quartile of fat intake were more likely to be younger, have achieved a higher level of education, have prevalent diabetes, and have higher intakes of total energy and preserved fish and shellfish than individuals in the lowest quartile of fat intake at baseline. Compared to male cohort members, female cohort members were less likely to be smokers or consume alcohol, have achieved a secondary level of education or higher, or be members of the Hokkien dialect. Although women tended to consume less energy overall than men, there were no notable differences in fat intakes between the two sexes.

Table 41. Demographics of male and female members of the Singapore Chinese Health Study stratified by quartile of fat intake.

	Men (n=27,293)				Women (n=34,028)			
Quartile of total fat intake	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Characteristic								
Person-years of follow-up	80,643	82,034	82,402	81,195	107,810	108,572	108,088	106,314
Mean age at baseline, y (SD)	57.73 ± 7.90	56.88 ± 7.98	56.32 ± 7.91	55.50 ± 7.84	58.43 ± 8.15	56.79 ± 7.97	55.52 ± 7.77	54.18 ± 7.55
Hokkien dialect (%)	55.6	56.6	55.8	55.6	52.3	52.4	52.4	51.5
Body mass index, kg/m^2 (%)								
<20	16	16	15	15	15	15	14	15
20-<24	56	53	53	50	57	55	54	53
24-<28	22	25	26	26	21	22	23	24
≥28	5	6	6	8	7	8	8	9
Secondary level of education or higher (%)	28	36	40	47	13	18	23	30
Smoking status (%)								
Never	39	42	44	43	89	92	92	92
Ex-smoker	22	21	21	21	3	2	3	2
Current	39	37	34	36	8	6	5	5
Weekly or daily alcohol consumption (%)	24	21	19	18	4	4	4	5
Self-reported history of ulcer at baseline (%)	4	5	5	5	2	2	2	2
Self-reported history of diabetes at baseline (%)	7	8	9	11	8	9	9	10
Total energy intake, kcal (mean ± SD)	1652.05 ± 516.53	1676.97 ± 568.34	1738.65 ± 591.93	1944.50 ± 703.68	1272.77 ± 420.00	1318.72 ± 393.60	1421.63 ± 452.47	1582.25 ± 548.32
Total fat intake, % kcal (mean ± SD)	17.09 ± 2.71	22.58 ± 1.14	26.32 ± 1.10	31.69 ± 2.76	18.60 ± 2.71	23.84 ± 1.07	24.47 ± 1.09	32.64 ± 2.65
Total preserved fish and shellfish intake, g/kcal (mean ± SD)	1.32 ± 1.61	1.70 ± 1.76	2.02 ± 2.15	2.56 ± 2.66	1.26 ± 1.63	1.61 ± 1.81	1.89 ± 2.16	2.36 ± 2.49

There were no significant associations between fish intake and the risk of gastric cancer in the overall cohort (Table 42). Among men, there was a borderline statistically significant inverse trend between total fish and shellfish intake and the risk of gastric cancer ($p = 0.14$), with a significant 31% decrease in risk in the second quartile of exposure. There were no statistically significant trends or HRs among women with regards to fish intake.

Fat intakes were not significantly associated with gastric cancer risk among the entire cohort (Table 43). There were borderline significant positive trends between animal fat intake ($p = 0.12$) and cholesterol ($p = 0.08$) intake and gastric cancer risk among male cohort members, but no HRs were statistically significant. Among women, there was a significant inverse association between red meat fat intake and gastric cancer risk ($p = 0.005$), with the highest quartile of intake imparting a 46% decreased risk of gastric cancer.

In the overall cohort, there were borderline statistically significant inverse associations between total carotenoids ($p = 0.11$), β-cryptoxanthin ($p = 0.06$), and vitamin C ($p = 0.10$) and the risk of gastric cancer (Table 44). The second quartiles of total carotenoids or vitamin C exposure were associated with significant 24% and 22% decreases in gastric cancer risk, respectively, while the highest quartile of β-cryptoxanthin intake conferred a significant 23% reduction in gastric cancer risk. Among male cohort members, the inverse trend between β-cryptoxanthin and gastric cancer risk was statistically significant ($p = 0.04$), but no individual HRs achieved statistical significance. In women, the inverse trend between total carotenoids and gastric cancer

Table 42. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fish intake and the risk of gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
Total fish and shellfish									
Q1	17.60 ± 6.04	141	1.00 (ref)	16.49 ± 5.49	92	1.00 (ref)	18.67 ± 6.34	49	1.00 (ref)
Q2	29.10 ± 3.25	120	0.86 (0.68—1.10)	27.61 ± 2.81	65	0.69 (0.50—0.95)	30.46 ± 3.02	55	1.19 (0.81—1.75)
Q3	39.46 ± 3.80	127	0.90 (0.71—1.15)	37.46 ± 3.26	79	0.83 (0.61—1.12)	41.06 ± 3.43	48	1.02 (0.69—1.53)
Q4	55.58 ± 13.99	131	0.90 (0.71—1.15)	53.05 ± 13.55	81	0.83 (0.61—1.12)	57.42 ± 14.01	50	1.03 (0.69—1.53)
***p* for trend**			0.66			0.14			0.79
Fresh fish and shellfish									
Q1	15.91 ± 5.73	139	1.00 (ref)	14.80 ± 5.18	90	1.00 (ref)	17.08 ± 6.02	49	1.00 (ref)
Q2	27.27 ± 3.24	121	0.88 (0.69—1.12)	25.73 ± 2.76	68	0.73 (0.53—1.00)	28.60 ± 3.00	53	1.14 (0.77—1.68)
Q3	37.54 ± 3.81	126	0.90 (0.71—1.15)	35.45 ± 3.23	75	0.80 (0.59—1.08)	39.19 ± 3.41	51	1.09 (0.73—1.61)
Q4	53.50 ± 14.01	133	0.93 (0.73—1.18)	50.85 ± 13.55	84	0.88 (0.65—1.19)	55.52 ± 14.02	49	0.99 (0.67—1.48)
***p* for trend**			0.74			0.23			0.88
Preserved fish and shellfish									
Q1	0.16 ± 0.13	137	1.00 (ref)	0.19 ± 0.15	84	1.00 (ref)	0.15 ± 0.12	53	1.00 (ref)
Q2	0.80 ± 0.25	12	0.91 (0.71—1.16)	0.88 ± 0.24	75	0.89 (0.65—1.22)	0.73 ± 0.23	49	0.95 (0.64—1.40)
Q3	1.79 ± 0.37	121	0.88 (0.69—1.13)	1.85 ± 0.36	71	0.83 (0.60—1.13)	1.73 ± 0.36	50	0.99 (0.67—1.47)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
Q4	3.75 ± 2.57	137	1.01 (0.80—1.29)	3.83 ± 2.59	87	1.02 (0.75—1.38)	3.69 ± 2.56	50	1.02 (0.69—1.50)
***p* for trend**			0.60			0.52			0.99
Fish, fresh and preserved									
Q1	15.03 ± 5.50	135	1.00 (ref)	13.95 ± 4.96	85	1.00 (ref)	16.11 ± 5.78	50	1.00 (ref)
Q2	25.93 ± 3.13	135	1.00 (0.79—1.27)	24.44 ± 2.69	81	0.92 (0.67—1.24)	27.21 ± 2.86	54	1.13 (0.77—1.66)
Q3	35.90 ± 3.74	110	0.80 (0.62—1.02)	33.82 ± 3.13	69	0.76 (0.55—1.04)	37.57 ± 3.37	41	0.84 (0.56—1.27)
Q4	51.73 ± 13.95	139	0.97 (0.76—1.23)	53.06 ± 13.52	82	0.88 (0.65—1.20)	53.69 ± 13.93	57	1.11 (0.76—1.63)
***p* for trend**			0.24			0.40			0.47
Shellfish, fresh and preserved									
Q1	0.69 ± 0.41	153	1.00 (ref)	0.81 ± 0.43	89	1.00 (ref)	0.61 ± 0.38	64	1.00 (ref)
Q2	1.92 ± 0.36	129	0.93 (0.74—1.18)	2.03 ± 0.34	72	0.88 (0.65—1.20)	1.82 ± 0.35	57	1.02 (0.71—1.46)
Q3	3.26 ± 0.50	111	0.85 (0.67—1.09)	3.34 ± 0.47	74	0.96 (0.70—1.31)	3.19 ± 0.51	37	0.70 (0.47—1.06)
Q4	6.06 ± 3.19	126	0.98 (0.77—1.25)	6.02 ± 2.87	82	1.05 (0.77—1.43)	6.11 ± 3.42	44	0.87 (0.59—1.30)
***p* for trend**			0.60			0.74			0.30

*Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 43. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fat intakes and the risk of gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
Total Fat									
Q1	18.57 ± 2.81	143	1.00 (ref)	17.71 ± 2.71	82	1.00 (ref)	19.29 ± 2.71	61	1.00 (ref)
Q2	23.33 ± 1.26	134	1.03 (0.81—1.30)	22.62 ± 1.14	82	1.06 (0.78—1.45)	23.88 ± 1.07	52	0.98 (0.67—1.41)
Q3	26.93 ± 1.23	136	1.13 (0.89—1.43)	26.27 ± 1.10	86	1.18 (0.87—1.59)	27.44 ± 1.09	50	1.06 (0.73—1.56)
Q4	31.60 ± 2.74	106	0.99 (0.76—1.28)	31.05 ± 2.76	67	1.00 (0.72—1.39)	32.02 ± 2.65	39	0.97 (0.64—1.47)
***p* for trend**			0.70			0.70			0.97
Saturated Fat									
Q1	5.92 ± 1.01	138	1.00 (ref)	5.76 ± 0.99	79	1.00 (ref)	6.06 ± 1.01	59	1.00 (ref)
Q2	7.93 ± 0.51	132	1.06 (0.84—1.35)	7.77 ± 0.49	83	1.16 (0.85—1.58)	8.16 ± 0.49	49	0.94 (0.64—1.38)
Q3	9.61 ± 0.54	135	1.16 (0.91—1.47)	9.41 ± 0.52	82	1.20 (0.88—1.63)	9.75 ± 0.52	53	1.12 (0.76—1.63)
Q4	11.85 ± 1.33	114	1.06 (0.82—1.37)	11.67 ± 1.34	73	1.14 (0.82—1.58)	12.01 ± 1.31	41	0.97 (0.64—1.46)
***p* for trend**			0.69			0.69			0.84
MUFA									
Q1	6.10 ± 0.96	137	1.00 (ref)	5.87 ± 0.93	83	1.00 (ref)	6.30 ± 0.93	54	1.00 (ref)
Q2	7.79 ± 0.43	130	1.04 (0.82—1.32)	7.59 ± 0.40	74	0.94 (0.69—1.29)	7.94 ± 0.39	56	1.19 (0.82—1.73)
Q3	9.11 ± 0.43	136	1.16 (0.91—1.47)	8.94 ± 0.41	83	1.11 (0.81—1.50)	9.25 ± 0.40	53	1.25 (0.85—1.84)
Q4	10.88 ± 1.09	116	1.09 (0.84—1.40)	10.73 ± 1.10	77	1.10 (0.80—1.51)	10.99 ± 1.07	39	1.05 (0.68—1.61)
***p* for trend**			0.65			0.70			0.64

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
PUFA									
Q1	3.24 ± 0.55	163	1.00 (ref)	3.09 ± 0.52	96	1.00 (ref)	3.38 ± 0.54	67	1.00 (ref)
Q2	4.26 ± 0.31	117	0.79 (0.63—1.01)	4.08 ± 0.25	74	0.82 (0.61—1.12)	4.42 ± 0.27	43	0.75 (0.51—1.10)
Q3	5.27 ± 0.43	118	0.85 (0.67—1.09)	5.03 ± 0.34	68	0.79 (0.57—1.08)	5.45 ± 0.39	50	0.95 (0.65—1.38)
Q4	7.30 ± 1.48	121	0.90 (0.70—1.14)	6.90 ± 1.38	79	0.94 (0.69—1.27)	7.62 ± 1.49	42	0.81 (0.55—1.21)
***p* for trend**			0.27			0.40			0.44
Total n-3									
Q1	0.36 ± 0.055	140	1.00 (ref)	0.34 ± 0.051	83	1.00 (ref)	0.38 ± 0.054	57	1.00 (ref)
Q2	0.45 ± 0.030	128	0.97 (0.76—1.23)	0.43 ± 0.022	71	0.87 (0.63—1.20)	0.47 ± 0.023	57	1.11 (0.76—1.60)
Q3	0.53 ± 0.033	131	0.99 (0.72—1.18)	0.51 ± 0.025	91	1.09 (0.80—1.46)	0.55 ± 0.026	40	0.81 (0.54—1.22)
Q4	0.67 ± 0.18	120	0.92 (0.72—1.18)	0.64 ± 0.16	72	0.87 (0.63—1.19)	0.69 ± 0.19	48	1.01 (0.68—1.48)
***p* for trend**			0.93			0.40			0.52
Marine n-3									
Q1	0.092 ± 0.032	146	1.00 (ref)	0.087 ± 0.029	90	1.00 (ref)	0.097 ± 0.033	56	1.00 (ref)
Q2	0.15 ± 0.017	116	0.81 (0.63—1.03)	0.15 ± 0.015	68	0.74 (0.54—1.02)	0.16 ± 0.016	48	0.91 (0.62—1.34)
Q3	0.21 ± 0.019	131	0.90 (0.72—1.14)	0.20 ± 0.017	81	0.87 (0.64—1.18)	0.21 ± 0.018	50	0.93 (0.64—1.37)
Q4	0.29 ± 0.072	126	0.84 (0.66—1.07)	0.28 ± 0.071	78	0.82 (0.60—1.11)	0.30 ± 0.072	48	0.87 (0.59—1.28)
***p* for trend**			0.33			0.29			0.91
Non-marine n-3									

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
Q1	0.21 ± 0.033	157	1.00 (ref)	0.21 ± 0.030	92	1.00 (ref)	0.22 ± 0.032	65	1.00 (ref)
Q2	0.28 ± 0.019	123	0.86 (0.68—1.09)	0.26 ± 0.015	78	0.89 (0.66—1.20)	0.29 ± 0.015	45	0.81 (0.55—1.19)
Q3	0.33 ± 0.021	114	0.82 (0.64—1.04)	0.31 ± 0.017	71	0.82 (0.60—1.12)	0.34 ± 0.018	43	0.81 (0.55—1.20)
Q4	0.42 ± 0.18	125	0.91 (0.72—1.16)	0.40 ± 0.16	76	0.86 (0.63—1.18)	0.44 ± 0.19	49	0.97 (0.66—1.42)
***p* for trend**			0.38			0.63			0.58
Total n-6									
Q1	2.82 ± 0.49	161	1.00 (ref)	2.69 ± 0.46	93	1.00 (ref)	2.95 ± 0.48	68	1.00 (ref)
Q2	3.75 ± 0.29	121	0.83 (0.66—1.06)	3.59 ± 0.23	78	0.89 (0.66—1.21)	3.89 ± 0.25	43	0.74 (0.50—1.09)
Q3	4.70 ± 0.41	114	0.84 (0.66—1.07)	4.47 ± 0.32	65	0.78 (0.57—1.08)	4.87 ± 0.38	49	0.91 (0.63—1.32)
Q4	6.64 ± 1.40	123	0.92 (0.72—1.17)	6.26 ± 1.30	81	0.99 (0.73—1.35)	6.95 ± 1.40	42	0.80 (0.54—1.18)
***p* for trend**			0.37			0.41			0.42
Total animal fat									
Q1	4.57 ± 1.27	152	1.00 (ref)	4.82 ± 1.30	88	1.00 (ref)	4.41 ± 1.21	64	1.00 (ref)
Q2	7.14 ± 0.70	121	0.87 (0.68—1.10)	7.49 ± 0.64	74	0.90 (0.66—1.23)	6.87 ± 0.61	47	0.83 (0.57—1.22)
Q3	9.31 ± 0.78	125	0.97 (0.76—1.23)	9.70 ± 0.71	67	0.86 (0.62—1.18)	8.99 ± 0.66	58	1.14 (0.79—1.64)
Q4	12.40 ± 2.23	121	1.02 (0.80—1.30)	12.93 ± 2.23	88	1.22 (0.90—1.66)	11.93 ± 2.12	33	0.72 (0.46—1.10)
***p* for trend**			0.60			0.12			0.14
Total red meat fat									
Q1	1.08 ± 0.49	153	1.00 (ref)	1.25 ± 0.51	75	1.00 (ref)	0.96 ± 0.44	78	1.00 (ref)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)***
Q2	2.20 ± 0.34	123	0.85 (0.67—1.07)	2.40 ± 0.30	74	1.04 (0.76—1.44)	2.05 ± 0.29	49	0.67 (0.47—0.96)
Q3	3.28 ± 0.43	117	0.84 (0.66—1.06)	3.52 ± 0.38	77	1.11 (0.80—1.52)	3.08 ± 0.36	40	0.58 (0.39—0.85)
Q4	5.08 ± 1.58	126	0.94 (0.74—1.20)	5.40 ± 1.63	91	1.36 (0.99—1.86)	4.81 ± 1.47	35	0.54 (0.36—0.81)
***p* for trend**			0.39			0.21			0.005
Total plant fat									
Q1	11.65 ± 2.02	139	1.00 (ref)	10.74 ± 1.76	91	1.00 (ref)	12.61 ± 1.85	48	1.00 (ref)
Q2	15.06 ± 1.23	126	0.96 (0.75—1.22)	14.03 ± 0.80	70	0.78 (0.57—1.07)	15.92 ± 0.79	56	1.30 (0.88—1.91)
Q3	17.80 ± 1.28	134	1.09 (0.86—1.38)	16.71 ± 0.83	76	0.88 (0.65—1.20)	18.63 ± 0.86	58	1.47 (1.00—2.17)
Q4	21.74 ± 2.92	120	1.03 (0.81—1.33)	20.48 ± 2.72	80	0.98 (0.72—1.33)	22.52 ± 2.76	40	1.10 (0.72—1.68)
***p* for trend**			0.78			0.41			0.20
Total cholesterol									
Q1	62.53 ± 15.42	148	1.00 (ref)	62.88 ± 15.37	84	1.00 (ref)	62.30 ± 15.45	64	1.00 (ref)
Q2	92.04 ± 7.33	112	0.84 (0.66—1.08)	93.16 ± 7.37	63	0.82 (0.59—1.14)	90.98 ± 7.16	49	0.88 (0.60—1.28)
Q3	116.95 ± 8.35	127	1.02 (0.81—1.30)	118.83 ± 8.56	81	1.10 (0.81—1.50)	115.49 ± 7.85	46	0.91 (0.62—1.34)
Q4	156.29 ± 42.14	132	1.12 0.88—1.42)	161.28 ± 47.29	89	1.25 (0.93—1.70)	152.25 ± 36.47	43	0.92 (0.62—1.37)
***p* for trend**			0.18			0.08			0.92

[*]Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 44. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of antioxidant intake and the risk of gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
Total selenium (μg/kcal)									
Q1	53.81 ± 5.07	135	1.00 (ref)	53.76 ± 4.85	79	1.00 (ref)	53.83 ± 5.25	56	1.00 (ref)
Q2	61.56 ± 1.88	109	0.79 (0.61—1.01)	61.09 ± 1.75	63	0.78 (0.56—1.09)	61.96 ± 1.90	46	0.80 (0.54—1.18)
Q3	67.82 ± 2.11	138	0.97 (0.76—1.23)	67.11 ± 1.94	88	1.05 (0.78—1.43)	68.37 ± 2.07	50	0.85 (0.58—1.25)
Q4	77.31 ± 8.75	137	0.92 (0.72—1.17)	76.03 ± 7.95	87	1.01 (0.74—1.37)	78.19 ± 9.23	50	0.79 (0.54—1.16)
***p* for trend**			0.27			0.30			0.60
Total carotenoids (μg/kcal)									
Q1	1771.00 ± 499.15	173	1.00 (ref)	1569.97 ± 430.42	101	1.00 (ref)	1980.15 ± 476.48	72	1.00 (ref)
Q2	2795.98 ± 376.33	118	0.76 (0.60—0.96)	2524.97 ± 256.31	74	0.79 (0.58—1.07)	3045.52 ± 283.47	44	0.69 (0.48—1.01)
Q3	3844.34 ± 487.95	116	0.82 (0.64—1.03)	3486.02 ± 340.25	77	0.89 (0.66—1.21)	4125.13 ± 385.84	39	0.67 (0.45—1.00)
Q4	5811.57 ± 1948.95	112	0.87 (0.69—1.11)	5265.59 ± 1754.35	65	0.84 (0.61—1.15)	6201.37 ± 1980.48	47	0.89 (0.61—1.30)
***p* for trend**			0.11			0.46			0.12
α-carotene (μg/kcal)									
Q1	29.86 ± 20.52	148	1.00 (ref)	20.07 ± 13.44	95	1.00 (ref)	42.03 ± 21.37	53	1.00 (ref)
Q2	85.81 ± 22.09	131	0.89 (0.66—1.07)	68.89 ± 13.81	77	0.78 (0.58—1.06)	100.43 ± 16.50	54	1.05 (0.72—1.53)
Q3	153.81 ±	121	0.84	126.96 ±	75	0.77	176.40 ±	46	0.93

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
	36.78		(0.66—1.07)	23.53		(0.57—1.05)	30.25		(0.62—1.38)
Q4	332.95 ± 224.87	119	0.88 (0.69—1.13)	278.20 ± 187.03	70	0.76 (0.56—1.04)	375.39 ± 239.39	49	1.07 (0.73—1.59)
***p* for trend**			0.52			0.23			0.90
β-carotene (μg/kcal)									
Q1	635.74 ± 201.97	151	1.00 (ref)	538.81 ± 160.12	90	1.00 (ref)	747.12 ± 186.11	61	1.00 (ref)
Q2	1038.41 ± 171.11	146	1.04 (0.83—1.31)	898.74 ± 97.04	91	1.07 (0.80—1.43)	1164.68 ± 116.11	55	0.98 (0.68—1.41)
Q3	1465.99 ± 232.05	105	0.80 (0.62—1.03)	1275.45 ± 132.95	67	0.83 (0.60—1.14)	1626.14 ± 164.74	38	0.73 (0.48—1.09)
Q4	2299.51 ± 880.12	117	0.96 (0.75—1.22)	1975.03 ± 741.98	69	0.91 (0.66—1.25)	2521.20 ± 892.44	48	1.00 (0.68—1.47)
***p* for trend**			0.19			0.42			0.39
β-cryptoxanthin (μg/kcal)									
Q1	12.06 ± 14.35	160	1.00 (ref)	13.28 ± 14.40	86	1.00 (ref)	10.73 ± 14.30	74	1.00 (ref)
Q2	71.46 ± 17.14	120	0.88 (0.69—1.12)	69.13 ± 15.88	77	0.99 (0.73—1.35)	73.50 ± 17.87	43	0.72 (0.49—1.06)
Q3	142.32 ± 28.18	138	1.07 (0.85—1.36)	136.38 ± 26.63	94	1.26 (0.93—1.69)	147.06 ± 28.41	44	0.80 (0.54—1.17)
Q4	323.55 ± 283.03	101	0.77 (0.60—1.00)	309.38 ± 254.88	60	0.78 (0.55—1.09)	333.47 ± 302.25	41	0.74 (0.50—1.09)
***p* for trend**			0.06			0.04			0.28
Lycopene (μg/kcal)									
Q1	90.53 ± 61.09	173	1.00 (ref)	105.40 ± 65.89	101	1.00 (ref)	80.26 ± 54.58	72	1.00 (ref)
Q2	312.87 ±	117	0.79	345.30 ±	74	0.83	287.52 ±	43	0.71

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
	79.75		(0.62—1.00)	78.00		(0.62—1.13)	72.27		(0.49—1.04)
Q3	627.01 ± 119.05	124	0.91 (0.72—1.16)	657.09 ± 111.43	78	0.95 (0.71—1.29)	603.63 ± 119.41	46	0.85 (0.58—1.24)
Q4	1311.77 ± 1210.93	105	0.90 (0.70—1.15)	1396.34 ± 1196.84	64	0.92 (0.66—1.26)	1245.72 ± 1219.55	41	0.85 (0.57—1.27)
***p* for trend**			0.26			0.70			0.38
Lutein (μg/kcal)									
Q1	597.01 ± 173.40	141	1.00 (ref)	520.16 ± 139.35	87	1.00 (ref)	688.74 ± 163.54	54	1.00 (ref)
Q2	939.52 ± 143.27	145	1.12 (0.89—1.42)	825.32 ± 81.85	82	1.01 (0.75—1.37)	1045.90 ± 97.82	63	1.28 (0.89—1.84)
Q3	1296.07 ± 191.42	116	0.95 (0.74—1.21)	1132.96 ± 110.31	76	1.00 (0.73—1.36)	1427.47 ± 134.42	40	0.85 (0.56—1.28)
Q4	1949.59 ± 639.28	117	0.99 (0.77-1.27)	1684.55 ± 518.06	72	0.97 (0.71—1.33)	2128.98	45	0.99 (0.66—1.48)
***p* for trend**			0.56			0.99			0.21
Total vitamin C (mg/kcal)									
Q1	21.66 ± 6.76	169	1.00 (ref)	19.99 ± 6.50	93	1.00 (ref)	23.02 ± 6.68	76	1.00 (ref)
Q2	39.63 ± 5.71	114	0.78 (0.61—0.99)	37.35 ± 4.99	71	0.83 (0.61—1.14)	41.60 ± 5.50	43	0.69 (0.47—1.01)
Q3	60.24 ± 8.33	132	1.00 (0.80—1.27)	56.55 ± 6.84	91	1.16 (0.87—1.56)	63.40 ± 8.09	41	0.74 (0.50—1.09)
Q4	102.73 ± 151.60	104	0.83 (0.65—1.07)	94.37 ± 127.18	62	0.82 (0.59—1.14)	108.74 ± 167.54	42	0.81 (0.55—1.20)
***p* for trend**			0.10			0.09			0.21
Total vitamin E (mg α-tocopherol equivalents/kcal)									
Q1	2.59 ± 0.47	151	1.00 (ref)	2.38 ± 0.40	92	1.00 (ref)	2.84 ± 0.43	59	1.00 (ref)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
Q2	3.41 ± 0.31	130	0.95 (0.75—1.21)	3.14 ± 0.19	76	0.89 (0.66—1.21)	3.64 ± 0.20	54	1.05 (0.72—1.52)
Q3	4.10 ± 0.34	124	0.99 (0.78—1.25)	3.80 ± 0.21	72	0.89 (0.65—1.21)	4.33 ± 0.23	52	1.13 (0.77—1.64)
Q4	5.25 ± 64.00	114	0.96 (0.75—1.23)	4.85 ± 54.66	77	0.99 (0.72—1.34)	5.49 ± 70.52	37	0.87 (0.57—1.33)
***p* for trend**			0.98			0.81			0.69
Total soy isoflavones (mg/kcal)									
Q1	3.63 ± 1.65	143	1.00 (ref)	3.25 ± 1.45	91	1.00 (ref)	4.00 ± 1.72	52	1.00 (ref)
Q2	7.74 ± 1.35	129	0.94 (0.74—1.20)	7.02 ± 1.07	72	0.80 (0.59—1.10)	8.37 ± 1.24	57	1.18 (0.81—1.72)
Q3	12.24 ± 1.87	136	1.03 (0.81—1.31)	11.12 ± 1.46	84	0.96 (0.71—1.29)	13.15 ± 1.66	52	1.15 (0.78—1.70)
Q4	20.72 ± 9.85	111	0.85 (0.66—1.10)	19.12 ± 8.76	70	0.80 (0.58—1.09)	22.00 ± 10.39	41	0.95 (0.63—1.43)
***p* for trend**			0.48			0.35			0.64
Genistein (mg/kcal)									
Q1	1.70 ± 0.76	142	1.00 (ref)	1.52 ± 0.67	91	1.00 (ref)	1.88 ± 0.80	51	1.00 (ref)
Q2	3.58 ± 0.62	136	1.01 (0.79—1.27)	3.24 ± 0.49	77	0.87 (0.64—1.18)	3.87 ± 0.56	59	1.25 (0.86—1.83)
Q3	5.63 ± 0.85	129	0.98 (0.77—1.25)	5.10 ± 0.66	78	0.88 (0.65—1.19)	6.04 ± 0.75	51	1.15 (0.78—1.71)
Q4	9.46 ± 4.37	112	0.87 (0.68—1.12)	8.72 ± 3.90	71	0.81 (0.59—1.11)	10.04 ± 4.59	41	0.97 (0.64—1.47)
***p* for trend**			0.65			0.60			0.53
Daidzein (mg/kcal)									
Q1	1.69 ± 0.78	143	1.00 (ref)	1.52 ± 0.69	89	1.00 (ref)	1.86 ± 0.81	54	1.00 (ref)
Q2	3.65 ± 0.64	130	0.95	3.31 ± 0.52	73	0.84	3.96 ± 0.59	57	1.14

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
			(0.75—1.21)			(0.61—1.14)			(0.79—1.66)
Q3	5.81 ± 0.90	135	1.03 (0.81—1.30)	5.28 ± 0.70	86	1.01 (0.75—1.36)	6.24 ± 0.80	49	1.05 (0.71—1.54)
Q4	9.92 ± 4.84	111	0.86 (0.67—1.10)	9.14 ± 4.29	69	0.80 (0.58—1.10)	10.55 ± 5.12	42	0.94 (0.62—1.41)
***p* for trend**			0.50			0.35			0.79
Glycitein (mg/kcal)									
Q1	0.24 ± 0.11	146	1.00 (ref)	0.21 ± 0.096	91	1.00 (ref)	0.26 ± 0.11	55	1.00 (ref)
Q2	0.51 ± 0.087	127	0.91 (0.72—1.15)	0.46 ± 0.070	72	0.81 (0.59—1.10)	0.55 ± 0.080	55	1.08 (0.74—1.57)
Q3	0.80 ± 0.12	137	1.02 (0.81—1.29)	0.73 ± 0.096	86	0.99 (0.73—1.33)	0.86 ± 0.11	51	1.07 (0.73—1.57)
Q4	1.36 ± 0.65	109	0.82 (0.64—1.05)	1.25 ± 0.58	68	0.77 (0.56—1.06)	1.44 ± 0.69	41	0.89 (0.59—1.35)
***p* for trend**			0.30			0.24			0.80
Total isothiocyanate (μmol/kcal)									
Q1	2.39 ± 0.84	140	1.00 (ref)	2.06 ± 0.71	90	1.00 (ref)	2.71 ± 0.83	50	1.00 (ref)
Q2	4.26 ± 0.68	135	1.04 (0.82—1.32)	3.79 ± 0.48	72	0.85 (0.62—1.16)	4.68 ± 0.54	63	1.37 (0.94—1.99)
Q3	6.25 ± 0.92	134	1.09 (0.86—1.39)	5.59 ± 0.66	86	1.09 (0.81—1.47)	6.77 ± 0.75	48	1.09 (0.73—1.62)
Q4	10.08 ± 4.36	110	0.94 (0.73—1.21)	9.08 ± 3.79	69	0.90 (0.66—1.24)	10.81 ± 4.58	41	0.97 (0.64—1.48)
***p* for trend**			0.67			0.40			0.26

*Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

risk remained borderline statistically significant ($p = 0.12$), and individuals in the third quartile of intake had a significant 33% reduction of gastric cancer risk. There were no significant associations between selenium, lycopene, lutein, soy isoflavones, or isothiocyanates and gastric cancer risk in the cohort.

Green tea, black tea, and coffee consumption were not significantly associated with gastric cancer risk within the overall cohort or among men (Table 45). In women, there was a borderline statistically significant trend between coffee consumption and gastric cancer risk ($p = 0.07$), and daily coffee consumption was associated with a significant 34% decrease in gastric cancer risk. There were no significant associations between tea consumption and gastric cancer risk within the cohort or among men or women.

Data from the above analyses stratified by gastric cancer site (cardia, non-cardia, or unspecified) are included in the appendix. Among men, there was a borderline statistically significant inverse association between fresh fish and shellfish intake and the risk of gastric cardia cancer (p for trend = 0.11), and the second quartile of intake was associated with a 60% decrease in risk (HR = 0.40, 95% CI 0.18—0.92). Increased total PUFA intake was borderline significantly associated with decreased gastric cardia cancer risk in the overall cohort and among women (p for trend = 0.06 and 0.15, respectively). There was also a borderline statistically significant inverse association between total n-3 intake and gastric cardia cancer risk among men (p for trend = 0.15), with the highest quartile of intake imparting a borderline significant 57% reduction in risk (HR = 0.43, 95% CI 0.17—1.06). Among the overall cohort, non-marine n-3 and total n-6 were

Table 45. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for tea and coffee intakes and the risk of gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
Black tea (cups/month)									
Q1	0.00	331	1.00 (ref)	0.00	187	1.00 (ref)	0.00	144	1.00 (ref)
Q2	2.00 ± 1.14	69	1.12 (0.86—1.45)	2.00 ± 1.14	39	0.98 (0.69—1.38)	2.00 ± 1.13	30	1.37 (0.92—2.03)
Q3	10.70 ± 4.48	47	0.84 (0.62—1.15)	10.70 ± 4.61	33	0.79 (0.55—1.15)	10.70 ± 4.30	14	0.95 (0.55—1.65)
Q4	30.00 ± 26.28	72	1.24 (0.95—1.60)	30.00 ± 27.73	58	1.25 (0.92—1.68)	30.00 ± 23.36	14	1.12 (0.64—1.95)
***p* for trend**			0.17			0.21			0.46
Green tea (cups/month)									
Q1	0.00	305	1.00 (ref)	0.00	179	1.00 (ref)	0.00	126	1.00 (ref)
Q2	2.00 ± 1.11	91	1.06 (0.84—1.34)	2.00 ± 1.11	47	0.87 (0.63—1.20)	2.00 ± 1.10	44	1.36 (0.96—1.92)
Q3	10.70 ± 4.28	51	0.96 (0.71—1.29)	10.70 ± 4.44	36	0.91 (0.64—1.31)	10.70 ± 4.09	15	0.99 (0.58—1.70)
Q4	30.00 ± 40.71	72	1.08 (0.83—1.41)	75.00 ± 42.47	55	1.04 (0.76—1.41)	30.00 ± 38.12	17	1.04 (0.62—1.74)
***p* for trend**			0.88			0.77			0.36
Any tea (cups/month)									
Q1	0.00	214	1.00 (ref)	0.00	115	1.00 (ref)	0.00	99	1.00 (ref)
Q2	4.00 ± 1.83	103	1.04 (0.82—1.32)	4.00 ± 1.87	51	0.84 (0.60—1.16)	4.00 ± 1.79	52	1.34 (0.95—1.87)
Q3	10.70 ± 4.96	72	0.89 (0.68—1.17)	12.70 ± 5.09	49	0.82 (0.59—1.15)	10.70 ± 4.80	23	0.98 (0.62—1.56)
Q4	40.70 ± 39.87	130	1.09 (0.87—1.37)	40.70 ± 41.56	102	1.05 (0.80—1.37)	34.30 ± 37.07	28	1.03 (0.67—1.57)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***	**Median intake ± SD**	**Cases, n**	**HR (95% CI)***
***p* for trend**			0.58			0.38			0.36
Coffee (cups/week)									
Never	0.00	101	1.00 (ref)	0.00	56	1.00 (ref)	0.00	45	1.00 (ref)
Monthly	0.46 ± 0.15	10	0.99 (0.52—1.90)	0.46 ± 0.15	6	1.07 (0.46—2.49)	0.46 ± 0.15	4	0.92 (0.33—2.56)
Weekly	2.50 ± 1.46	49	1.03 (0.73—1.45)	2.50 ± 1.45	24	0.88 (0.54—1.42)	2.50 ± 1.47	25	1.28 (0.78—2.09)
1 cup/day	7.02 ± 0.79	160	0.83 (0.64—1.06)	7.02 ± 0.83	97	0.99 (0.71—1.38)	7.02 ± 0.77	63	0.66 (0.45—0.97)
2-3 cups/day	17.56 ± 1.96	176	1.01 (0.79—1.29)	17.56 ± 1.97	119	1.12 (0.81—1.55)	17.56 ± 1.96	57	0.92 (0.62—1.36)
≥4 cups/day	31.60 ± 5.19	23	0.92 (0.58—1.46)	31.60 ± 4.68	15	0.89 (0.50—1.59)	31.60 ± 5.95	8	1.22 (0.57—2.61)
***p* for trend**			0.50			0.84			0.07

*Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

borderline significantly inversely associated with gastric cardia cancer risk (p for trend = 0.10 for both). Lastly, total plant fat intake showed a borderline statistically significant inverse trend regarding gastric cardia cancer risk among both men and women (p for trend = 0.15 and 0.12, respectively) but not in the overall cohort.

With respect to gastric non-cardia cancer risk, there was a borderline inverse association for all shellfish among women only (p for trend = 0.07), and women in the highest quartile of intake had a 41% reduction in risk (HR = 0.59, 95% CI 0.36—0.97). In the overall cohort and among women, increased consumption of saturated fat was associated with a borderline statistically significant increase in gastric non-cardia cancer risk (p for trend = 0.13 for both). Among men, there was a borderline significant—though somewhat U-shaped—trend between total n-3 intake and gastric non-cardia cancer risk (p for linear trend = 0.12; third quartile: HR = 1.26, 95% CI 0.88—1.80; fourth quartile: HR = 0.93, 95% CI 0.63—1.37). Among women, there was a statistically significant inverse trend between total red meat fat intake and gastric non-cardia cancer risk (p for trend = 0.005, fourth quartile: HR = 0.40, 95% CI 0.23—0.68) and a borderline significant positive trend between total plant fat intake and gastric non-cardia cancer risk (p for trend = 0.13). Women in the third quartile of plant fat consumption were at an 81% increased risk of gastric non-cardia cancer (HR = 1.81, 95% CI 1.11—2.96). Cholesterol intake was borderline statistically significantly positively associated with gastric non-cardia cancer risk among men only (p for trend = 0.15). Total selenium and lycopene intakes demonstrated a borderline significant inverse association with gastric non-cardia cancer risk within the overall cohort (p for trend = 0.07 for both).

There were no statistically significant associations between fish intake and the risk of unspecificed gastric cancer, which refers to cases that could not be classified as either cardia or non-cardia due to lack of a pathological examination. In men, there was a borderline significant positive trend between animal fat and unspecified gastric cancer risk (p for trend = 0.12), and among women, there was a borderline significant inverse trend between marine n-3 intake and unspecified gastric cancer risk (p for trend = 0.13). There was also a borderline inverse trend between consumption of total carotenoids and unspecified gastric cancer risk among female cohort members, with the second quartile of intake associated with a significant 62% reduction in risk (HR = 0.38, 95% CI 0.16—0.89, p for trend = 0.11). Male members of the cohort exhibited a borderline significant decreased risk of unspecified gastric cancer with increasing consumption of β-cryptoxanthin (p for trend = 0.07), and lutein intake was associated with a borderline significant inverse association among the overall cohort (p for trend = 0.06).

Notably, total soy isoflavones, genistein, daidzein, and glycitein were borderline statistically significantly inversely associated with unspecified gastric cancer risk among men only (p for trends = 0.06, 0.07, 0.07, and 0.09, respectively). Among both men and women, increased total isothiocyanate consumption was associated with a significantly decreased risk of unspecified gastric cancer (p for trends = 0.05 and 0.02, respectively); however, there remained no significant association when the cohort was analyzed as a whole.

Discussion

The absence of statistically significant associations between fish intake and n-3 fatty acid intake and gastric cancer risk does not support the hypothesis that increased consumption of these dietary components decrease the risk of gastric cancer among the Singapore Chinese population. The antioxidants vitamin C, total carotenoids, and β-cryptoxanthin exhibited borderline statistically significant inverse trends within the cohort with regards to gastric cancer risk. Among women, there was a borderline significant inverse association between coffee consumption and gastric cancer risk that will be further examined in the subsequent chapter. There was a significant inverse trend between red meat fat intake and gastric cancer risk among women.

The inability to detect an association between fish consumption and the risk of gastric cancer among Singapore Chinese individuals may be due to low consumption of fish rich in n-3 among this population. Although the FFQ used in the study has been validated, it does not include different variables for specific types of fish. The exposure variables used for fish (all fish and shellfish, fresh fish and shellfish, preserved fish and shellfish, and fresh and preserved fish) would include fish and shellfish relatively low in n-3, such as shrimp, pollock, and crab, as well as the cold water fatty fish, such as salmon, mackerel, and sardines, which are considered good sources of long-chain PUFA[2, 6]. Independent analyses for n-3 from both marine and other sources also yielded null results in general, but there was a borderline trend for decreased gastric cardia cancer risk with increased non-marine n-3 intake, possibly due to anti-inflammatory effects or transcriptional regulation[23, 25, 337]. In this population, non-

marine n-3 intake accounts for approximately 62% of total n-3 intake, and the primary sources of non-marine n-3 are grains, cooking oils, soy products, and legumes[338]. However, a similar trend was observed with total n-6 and gastric cardia cancer, suggesting that modulation of inflammation may not be the predominant mechanism for chemoprevention. Given that both n-3 and n-6 are susceptible to lipid peroxidation, which has been shown to have chemopreventive and chemostatic effects, it is possible that PUFA may reduce the risk of gastric cardia cancer by this mechanism[76, 98, 103, 107, 335]. But it is important to note that the results were not significant and multiple comparisons were conducted, and these results may be spurious.

Although several epidemiological studies have implicated the carotenoids α- and β-carotene and total retinol equivalents in the prevention of gastric cancer[339-341], this is one of the first analyses to suggest β-cryptoxanthin is negatively associated with gastric cancer risk, especially among men. In a nested case-control analysis from the European Prospective Investigation into Cancer and Nutrition study, plasma β-cryptoxanthin concentrations were significantly lower in gastric cancer cases compared to controls ($p = 0.01$), and there was a significant negative trend for gastric cancer odds with increasing plasma β-cryptoxanthin after adjusting for several variables including *H. pylori* infection status (p for trend = 0.006)[342]. More research will be needed to elucidate potential mechanisms by which β-cryptoxanthin may reduce gastric cancer risk, but its chemoprevention may be due to antioxidant activity and transcriptional regulation, similar to other carotenoids[343, 344]. Given that this borderline significant association

is observed only with unspecified gastric cancer and not specific cancer sites, it is challenging to interpret, and it is possible that this result is spurious.

One of the surprising results of these analyses was the significant negative association between red meat fat and gastric cancer risk among Singapore Chinese women. Red meat is a particularly rich source of saturated fat, and excessive cooking may result in the formation of pro-carcinogenic compounds[268, 320, 345, 346]. It seems likely that there may be an underlying compounding factor, such as socioeconomic status, that would confer a reduced risk of gastric cancer among frequent consumers of red meat. In the Singapore Chinese Health Study, education level is assessed as a surrogate for socioeconomic status, and among women there is a statistically significant interaction between red meat fat and education with regards to gastric cancer risk (p = 0.01). Women who consume red meat more frequently may have better access to health care and other preventive factors including diets lower in sodium and higher in fruits and vegetables. Given that this significant association remains only with regard to gastric non-cardia cancer risk, *H. pylori* infection likely contributes additional confounding. Indeed, in a nested case-control study from this cohort, the association between red meat and gastric cancer risk was no longer significant among women when *H. pylori* infection and chronic atrophic gastritis were included in the unconditional logistic regression model (OR = 0.71, 95% CI 0.24—2.11, fourth quartile versus first quartile, p for trend = 0.31, data not shown).

The inverse association between soy isoflavones and unspecified gastric cancer risk in male Singapore Chinese cohort members was not expected; however it is

supported by previous research. In a recent *in vitro* study of a human gastric cancer cell line, Tang et al.[347] demonstrated significant up-regulation of apoptosis with increasing concentrations of daidzein. Naginata et al.[348] detected a significant reduction in gastric cancer mortality among Japanese men consuming the highest tertile of soy products (HR = 0.50, 95% CI 0.26—0.93, *p* for trend = 0.03). In a Korean nested case-control study, Ko et al.[349] concluded that the highest plasma concentrations of genistein and daidzein significantly decreased the odds of gastric cancer (OR = 0.54, 95% CI 0.31—0.93 for genistein; OR = 0.21, 95% CI 0.08—0.58 for daidzein). However, in a Japanese prospective cohort study, Hara et al.[350] did not detect any significant associations between total soy isoflavone intake and gastric cancer. This study was the only one to report results by gastric cancer site—which did not differ. From the previous studies, it was not evident if the associations between soy isoflavones and a reduction in gastric cancer risk were site-specific, although given the countries in which the studies were conducted (Japan and Korea), it is likely that most gastric cancer cases affected the non-cardia portion of the stomach. In the present analysis, the association between soy isoflavones is only observed for unspecified gastric cancer among men; therefore, it is difficult to ascertain the biological mechanisms by which soy isoflavones may reduce the risk of gastric cancer.

Among both male and female cohort members, there were significant inverse associations between total isothiocyanate intake and unspecified gastric cancer risk. Isothiocyanates are produced in plants such as cruciferous vegetables (broccoli, cabbage, Brussels sprouts, etc.) as glucosinolates, which are converted into isothiocyanates when

the plant tissues are processed or chewed[155]. Isothiocyanates, such as SFN and I3C, have demonstrated chemopreventive effects in several experiments (Chapter III)[169, 176-178, 180, 186, 193, 197, 351]. Previously, SFN was shown to inhibit the growth and trigger the death of *H. pylori in vitro* and significantly decrease tumor multiplicity in a mouse model of gastric cancer[352]. In a prospective cohort of Chinese men, Moy et al.[353] concluded that men in the highest tertile of urinary isothiocyanate concentration were at a significant 34% reduction in gastric cancer risk (OR = 0.66, 95% CI 0.47—0.97, *p* for trend = 0.02), and this association was even stronger among men with homozygous deletion of glutathione *S*-transferase (GST) enzyme genes, suggesting that increased exposure to isothiocyanates enhanced gastric chemoprevention. These associations persisted even after adjustment for *H. pylori*, and the chemopreventive effects of isothiocyanates may function via xenobiotic metabolism alterations rather than *H. pylori* inhibition. However, as with the previous studies, this study did not report the distribution of gastric cancer sites among its cases. Given that the present results are seen only in unspecified gastric cancer and not overall gastric cancer, it is possible that the results are spurious.

As with all epidemiological research, there are a number of limitations to the present study. Dietary intake was assessed only at baseline and may have been subject to measurement error on the part of the subject or interviewer; cohort members may also have changed their dietary patterns substantially throughout the follow-up period, resulting in potential misclassification bias regarding dietary exposure. The number of comparisons made for these analyses also increased the risk for chance or spurious

findings, as previously mentioned. For the prospective analyses, no information regarding *H. pylori* infection or chronic atrophic gastritis status was included. These two important risk factors for gastric cancer may have contributed confounding or other biases to the results. Lastly, the lack of site determination for nearly 20% of gastric cancer cases precludes accurate interpretation of results for unspecified gastric cancers.

To conclude, there were no significant associations between total fish or fat intakes and the risk of gastric cancer among Singapore Chinese adults. There was evidence of inverse trends between antioxidant intakes and gastric cancer risk and coffee intake and gastric cancer risk among female members of the cohort. Additional analyses of the Singapore Chinese Health Study including increased follow-up time and gastric cancer cases may reveal statistically significant associations between fish, fat, and antioxidant intakes and gastric cancer risk in this population.

Chapter VIII: Daily coffee drinking reduces gastric cancer risk in Singapore Chinese women irrespective of *Helicobacter pylori* infection or chronic atrophic gastritis

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Introduction

Gastric cancer is the third leading cause of cancer death in men and fifth leading cause of cancer death in women worldwide[264]. The incidence of gastric cancer is especially high in Asian populations due in part to traditional diets rich in salt and fermented foods. In Singapore, the age-standardized gastric cancer incidence rates are relatively high. The average annual incidence was 21.5 and 10.8 per 100,000 from 1998 to 2002, among men and women, respectively[265, 266, 270]. Infection of the stomach with *Helicobacter pylori* (*H. pylori*) is a strong risk factor for gastric cancer and was classified as a group 1 human carcinogen in 1994, and there is substantial evidence that *H. pylori* infection significantly increases the risk for non-cardia gastric cancer[269, 271-273]. Given the higher prevalence of *H. pylori* infection in Asian countries compared to Western countries, rates of non-cardia gastric cancer are especially high in countries such as Singapore[354]. In addition to *H. pylori*, chronic atrophic gastritis, a condition of loss of glandular differentiation in the stomach tissue caused by an infection or autoimmune response, is considered a precancerous condition, and screening for serum pepsinogens leaked from gastric cells has been established as a sensitive technique to detect individuals at high-risk of gastric cancer[355, 356]. In a recent analysis of a prospective cohort study conducted in Japan, the sensitivity and specificity of this technique were 71.0% and 69.2%, respectively, among individuals over 40 years of age with no history of gastric cancer or gastrectomy[357].

Although *H. pylori* infection is a strong risk factor for non-cardia gastric cancer, certain dietary factors are associated with risk of both non-cardia and cardia gastric

cancer. Epidemiologic studies have shown that high salt intake interacts synergistically with *H. pylori* infection to increase the risk of non-cardia gastric cancer, while a few studies demonstrated a statistically significant protective effect of vitamin C or fresh fruit for all gastric cancers regardless of *H. pylori* status[271, 273, 275, 301]. Additionally, consumption of allium vegetables, such as garlic and onions, has been inversely associated with risk of gastric cancer[266]. However, there are limited data addressing gastric cancer risk modulation by beverage sources of antioxidants, such as coffee.

Coffee is one of the most frequently consumed beverages worldwide, and consumption rates are higher in Singapore compared to other Asian countries, possibly due its history as a British colony[358, 359]. Kahweol and cafestol are polyphenols in coffee that have demonstrated chemopreventive properties in cell and animal models of various cancers[360-364]. In Singapore, coffee grounds are typically boiled in water and then filtered through a muslin bag, a process that may result in brewed coffee that has higher concentrations of the diterpenes kahweol and cafestol relative to the use of a paper filter, as in most Western populations[359, 365].

In addition to providing diterpenes and antioxidants such as chlorogenic acids, coffee is a source of melanoidins, a heterogeneous group of compounds that has shown antimicrobial effects against *H. pylori in vitro*[366-368]. A meta-analysis of seven cohort and 16 case-control studies by Botelho et al.[369] concluded that coffee consumption was not associated with gastric cancer risk (OR = 0.97; 95% CI: 0.86, 1.09), but the studies included were conducted in the United States, Japan, and Europe only. To explore whether there are associations between coffee consumption and the risk of gastric cancer

in the Singapore Chinese population, we conducted prospective analyses with the Singapore Chinese Health Study database, a population-based prospective cohort of Chinese men and women living in Singapore. Furthermore, the availability of biospecimens from this cohort allowed for testing of *H. pylori* infection and atrophic gastritis prevalence to explore the interaction between these high-risk conditions and coffee consumption with regard to gastric cancer risk, for which data are currently lacking.

Materials and Methods

Singapore Chinese Health Study

The design and inclusionary and exclusionary criteria for the Singapore Chinese Health Study have been previously described[280]. Members of the cohort were selected from permanent residents or citizens of Singapore aged 45 to 74 years living in government housing estates and belonging to one of the two major Chinese dialect groups (Cantonese and Hokkien)[279]. At baseline, all cohort members completed an in-person interview with a trained study staff member that included a validated 165-item food frequency questionnaire (FFQ) that assessed coffee intake at nine defined levels: never or hardly ever, one to three times per month, once per week, two to three times per week, four to six times per week, once per day, two to three times per day, four to five times per day, and six or more times per day[279]. Because of the infrequent consumption of decaffeinated coffee among Singapore Chinese, only caffeinated coffee intake was assessed during the baseline interview. In concert with the FFQ validation,

the Singapore Food Composition Database was developed using food composition data from China, Malaysia, Taiwan, and Hawaii; from this database, mean daily consumption values for 96 dietary components including caffeine were ascertained for each cohort member[279].

We collected biospecimens from 3% of random samples of cohort participants beginning in 1994 and from all consenting subjects in 2000. By April 2005, biospecimens were obtained from 32,543 subjects (28,330 bloods; 4,400 buccal cells from refusals donating blood; 31,895 urines), representing a consent rate of approximately 60% after excluding death. All extracted components from the biospecimens have been stored in –80^{o}C freezers. The Singapore Chinese Health Study was approved by the Institutional Review Boards at the National University of Singapore and the University of Pittsburgh.

Cancer diagnoses and deaths from cancer were identified by linking cohort members to the Singapore Cancer Registry and the Singapore Registry of Births and Deaths. This comprehensive national registry was established in 1968, and as of April 2008, only 27 cases were lost to follow-up due to migration out of Singapore. We used data from the 27,293 men and 34,028 women who did not have a history of cancer diagnosis at baseline, based on self-report and computer-assisted record linkage analysis with the Singapore Cancer Registry. As of December 31, 2008 (an average follow-up of 12.3 years), 519 of these cohort members had been diagnosed with gastric cancer (ICD-10 16.0). Of these cases, 73 affected the gastric cardia (ICD-10 C16.0), 345 affected the gastric non-cardia (ICD-10 C16.1 – C16.8), and 101 were unspecified (ICD-10 C16.9).

Cox proportional hazards regression analysis of diet, environmental factors, and gastric cancer incidence

Person-years of follow-up were computed from the recruitment date to the date of gastric cancer diagnosis, death, migration, or December 31, 2008, whichever occurred first. A series of Cox proportional hazards regression models were performed to test the associations between coffee and gastric cancer incidence. Defined categories of coffee intake frequencies (cups/week) were evaluated as exposure variables. Quartiles of caffeine exposure were derived from the intake distribution among the entire cohort. To control for confounding, the following were included as covariates: sex (male/female), dialect group (Cantonese/Hokkien), age (years), interview year (1993-1995/1996-1998), education (less than secondary/secondary or higher), tobacco smoking (ever/never), alcohol use (ever/never), and body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2). Additionally, daily energy intake (kcal/day) and daily caffeine intake (mg/day) were included as covariates in the final model to control for dietary factors associated with coffee consumption.

Statistical analyses were conducted for the overall cohort, within men, and within women for all gastric cancers, cardia gastric cancers, non-cardia gastric cancers, and unspecified gastric cancers. Within women, stratified analyses were conducted by median length of follow-up (e.g., seven years) to test the effects of coffee on gastric cancer risk by length of follow-up. All hazard ratios (HR) and 95% confidence intervals (CI) reported were two-sided and computed relative to the lowest quartile or category of intake, and $\alpha = 0.05$ was set as the cutoff for determining statistical significance. The

linear tests for coffee-gastric cancer trends were computed base on ordinal values for the categories (0,1,2,3, etc.).

Given previous results from the cohort, demographic factors (sex and education level) and self-reported history of diabetes were tested for multiplicative interaction in the adjusted proportional hazards models. There were no significant interactions between sex, history of diabetes, or reproductive factors (number of live births, menopausal status) and coffee among women with regards to gastric cancer risk. Adjustment for additional dietary factors (i.e. dairy, protein, fruit and vegetable, and preserved meat and fish intakes) did not change the association between coffee and gastric cancer in the overall cohort or among either sexes, and adjustment for reproductive factors (i.e. age at first period, number of live births, and menopausal status) in the proportional hazards model did not affect the association for coffee and gastric cancer risk among women.

Selection of subjects for case-control analyses

Gastric cancer cases for the case-control study were selected from individuals diagnosed with cancer through December 31, 2008, who donated blood samples at baseline or during follow-up (n=142). After excluding nine cases due to missing specimens or incident gastric cancer at baseline, 133 gastric cancer cases were included in the analyses. Gastric cancer-free controls who donated a blood sample were matched on age at study enrollment (within three years), sex, dialect, date of baseline interview (within two years), and date of sample collection (within six months), and 389 matched controls were included. Unconditional logistic regression was performed to calculate

adjusted odds ratios (OR) and 95% CIs for coffee and gastric cancer with adjustment for *H. pylori* infection and atrophic gastritis status.

H. pylori *and chronic atrophic gastritis testing*

Serum samples were tested for anti-*H. pylori* antigens by Western blot (Helico Blot 2.1, MP Biomedicals, Singapore) (unpublished data). Subjects were classified as *H. pylori*-positive according to kit specifications, which have been validated with 96% sensitivity and 95% specificity by the manufacturer. Serum pepsinogen I (PG I) and II (PG II) were measured with a latex agglination turbidimetric immunoassay kit (LZ Test "Eiken" Pepsinogen I and II, Tokyo, Japan) and an automated analyzer (Siemens Advia 2400). Subjects were classified as positive for atrophic gastritis if PG I <70 ng/mL and PG I:II <3, as recommended by the manufacturer. All assays were conducted by lab personnel blinded to the case-control status in sets of three or four subjects containing at least one case.

Statistical analysis

SAS v9.2 (SAS Institute, Inc. Cary, NC) was used for statistical analyses. All *p* values were two-sided and considered statistically significant if <0.05.

Results

The distribution of demographic characteristics of cohort members by coffee intake frequency are presented overall and stratified by sex (Table 46). The proportions

of current or former smokers increased with increasing coffee intake, and alcohol consumption was also slightly higher among cohort members drinking four or more cups of coffee per day compared to those consuming coffee less frequently. Compared to individuals consuming coffee weekly or less, those who drank more than four cups of coffee per day were less likely to have achieved a secondary or higher level of education, and women were less likely than men to have completed secondary or higher education. Additionally, the proportions of women that had smoked cigarettes or consumed alcohol regularly were lower than the proportions among men. Among women, the distribution of selected reproductive factors (age at first period, number of live births, and menopausal status) was similar across categories of coffee consumption (data not shown).

Overall, there was a weak, statistically non-significant inverse association for daily versus never/monthly coffee intake and gastric cancer risk (Table 47). After stratification by sex, the inverse relationship was clearest among women. Compared with never/monthly coffee intake, consuming at least one cup of coffee per day was associated with a statistically significant 37% reduction in gastric cancer risk among women, independent of caffeine intake. Among women, caffeine intake had a suggestive inverse association at low levels (e.g., second and third quartiles versus first quartile), but there was no association with the highest versus the lowest quartile in relation to gastric cancer risk. The inverse association with coffee and gastric cancer risk among women was present regardless of subsite (cardia and non-cardia) (data not shown). There was no association among men with coffee and caffeine intake and gastric cancer risk, regardless of subsite.

Table 46. Distribution of selected baseline characteristics stratified by sex and coffee intake frequency

Demographic Characteristic (unit of measurement)	Men				Women			
	Coffee intake				Coffee intake			
	≤weekly	1 cup/day	2-3 cups/day	≥4 cups/day	≤weekly	1 cup/day	2-3 cups/day	≥4 cups/day
Person years of follow-up	91,753	100,265	115,621	18,632	129,208	172,831	117,255	11,489
Hokkien dialect (%)	54.8	55.6	56.0	62.9	50.8	51.4	53.8	62.8
Mean age at baseline , y (SD)	56.99 ± 8.32	56.98 ± 7.98	56.17 ± 7.67	55.30 ± 7.32	56.09 ± 8.24	56.44 ± 8.02	56.06 ± 7.76	56.38 ± 7.74
Body mass index, kg/m^2 (%)								
<20	13.6	15.6	17.0	20.1	15.9	14.1	14.4	15.3
20-<24	51.8	53.0	54.2	54.2	54.6	54.7	55.2	56.2
24-<28	27.5	25.3	23.1	20.5	21.5	23.3	22.5	20.3
≥28	7.1	6.2	5.8	5.2	8.0	7.9	7.9	8.2
Secondary level of education or higher (%)	43.1	37.8	34.9	33.9	26.1	19.4	17.0	15.3
Smoking status (%)								
Never	53.4	44.1	35.0	19.4	94.7	91.6	88.4	76.7
Ex-smoker	23.5	22.4	19.9	14.4	2.1	2.7	2.6	3.4
Current	23.1	33.5	45.1	66.2	3.2	5.8	9.1	20.0
Weekly or daily alcohol consumption (%)	16.7	22.3	22.5	21.8	3.3	4.3	5.6	8.2
Self-reported history of ulcer at baseline (%)	6.6	4.1	3.7	5.6	3.3	1.8	1.8	3.0
Self-reported history of diabetes at baseline (%)	10.9	8.9	7.0	6.0	10.8	9.3	7.5	5.0
Total energy intake, kcal (mean ± SD)	1697.34 ± 584.07	1702.37 ± 599.13	1815.74 ± 618.44	1928.99 ± 673.97	1368.51 ± 465.30	1366.00 ± 450.84	1466.24 ± 492.60	1567.88 ± 558.33
Total caffeine intake (mean ± SD)	72.74 ± 70.11	126.25 ± 54.48	248.65 ± 58.24	439.52 ± 77.52	43.60 ± 51.18	108.20 ± 42.68	230.82 ± 51.33	430.55 ± 81.98
Non tea drinkers, n (%)	1778 (22.8)	2780 (32.9)	3755 (39.4)	762 (49.9)	4489 (43.4)	6415 (47.0)	4760 (52.1)	554 (61.9)
Total protein intake, % kcal (mean ± SD)	15.1 ± 2.5	14.9 ± 2.4	14.7 ± 2.4	14.5 ± 2.5	15.8 ± 2.5	15.5 ± 2.5	15.1 ± 2.3	14.8 ± 2.4
Total dairy intake, g/kcal	46.61 ±	34.06 ±	30.89 ± 51.01	26.96 ±	70.10 ± 96.89	52.33 ± 81.36	43.67 ± 68.96	35.67 ±

Demographic Characteristic (unit of measurement)	Men					Women			
	Coffee intake					Coffee intake			
	≤weekly	1 cup/day	2-3 cups/day	≥4 cups/day		≤weekly	1 cup/day	2-3 cups/day	≥4 cups/day
(mean ± SD)	72.49	58.30		45.26					61.51
Total fruit intake, g/kcal (mean ± SD)	132.76 ± 96.82	129.04 ± 91.57	110.46 ± 83.10	92.57 ± 75.27		146.83 ± 108.67	142.98 ± 102.83	121.94 ± 92.63	97.28 ± 93.17
Total vegetable intake, g/kcal (mean ± SD)	67.29 ± 32.53	65.93 ± 30.33	61.51 ± 29.51	56.46 ± 27.57		82.54 ± 38.99	80.86 ± 36.01	74.44 ± 34.18	70.19 ± 34.33
Total preserved fish and shellfish intake, g/kcal (mean ± SD)	1.86 ± 2.16	1.87 ± 2.12	1.92 ± 2.09	2.13 ± 2.34		1.73 ± 2.12	1.75 ± 2.01	1.85 ± 2.06	2.13 ± 2.98
Total preserved meat intake, g/kcal (mean ± SD)	1.60 ± 2.49	1.60 ± 2.46	1.68 ± 2.52	1.91 ± 2.86		1.49 ± 2.50	1.51 ± 2.50	1.59 ± 2.34	1.79 ± 3.00
Nested case-control, n	97	101	127	10		55	86	41	5
H. pylori positive (%)	83.5	89.1	89.0	90.0		87.3	76.7	95.1	60.0
Current infection marker positive (%)	73.2	74.3	73.2	80.0		80.0	66.3	82.9	60.0
Atrophic gastritis positive (%)	16.5	15.8	23.6	20.0		18.2	15.1	22.0	0.0

Table 47. Coffee and caffeine intake and gastric cancer risk in the Singapore Chinese Health Study

	Overall		Men		Women	
	Cases, n	**HR (95% CI)**	**Cases, n**	**HR (95% CI)**	**Cases, n**	**HR (95% CI)**
Coffee intake[*]						
Never/monthly[1]	111	1.00	62	1.00	49	1.00
Weekly	49	1.02 (0.73 – 1.44)	24	0.87 (0.54 – 1.40)	25	1.27 (0.78 – 2.08)
Daily	160	0.82 (0.63 – 1.06)	97	0.98 (0.70 – 1.37)	63	0.65 (0.42 – 0.99)
2-3 cups/day	176	0.97 (0.67 – 1.41)	119	1.09 (0.70 – 1.72)	57	0.85 (0.42 – 1.69)
≥4 cups/day	23	0.85 (0.41 – 1.78)	15	0.85 (0.35 – 2.05)	8	1.03 (0.26 – 4.13)
p for trend		0.37		0.77		0.04
Never/monthly[2]	111	1.00	62	1.00	49	1.00
Weekly	49	1.02 (0.73 – 1.43)	24	0.87 (0.54 – 1.39)	25	1.24 (0.76 – 2.01)
≥Daily	359	0.85 (0.66 – 1.09)	231	1.03 (0.74 – 1.42)	128	0.63 (0.42 – 0.94)
p for trend		0.32		0.77		0.009
Caffeine intake[†,3]						
Q1 (lowest)	143	1.00	84	1.00	59	1.00
Q2	115	0.86 (0.68 – 1.10)	71	0.96 (0.70 – 1.32)	44	0.72 (0.49 – 1.07)
Q3	132	0.92 (0.73 – 1.17)	84	1.09 (0.80 – 1.48)	48	0.74 (0.51 – 1.09)
Q4 (highest)	129	1.03 (0.81 – 1.32)	78	1.04 (0.76 – 1.43)	51	1.08 (0.74 – 1.59)
p for trend		0.49		0.88		0.10

[*]Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), caffeine (mg/day), and total energy intake (kcal/day)

[†]Adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal)

[1]*p* for sex interaction = 0.33

[2]*p* for sex interaction = 0.11

[3]*p* for sex interaction = 0.73

We conducted stratified analyses by duration of follow-up to investigate whether the relationship between coffee intake and gastric cancer was consistent with an early- or late-acting effect along the carcinogenesis pathway (Table 48). A statistically significant inverse association was confined to those with a longer duration of follow-up (e.g., ≥7 years). These findings are consistent with an early-acting protective effect of coffee intake on gastric cancer risk in women. The exclusion of cases diagnosed within the first two years of follow-up did not appreciably change the association between coffee and gastric cancer (at least daily versus less than daily: HR = 0.59; 95% CI: 0.39, 0.91, *p* for trend = 0.01, data not shown).

Table 48. Coffee intake in relation to gastric cancer risk among female members of the Singapore Chinese Health Study by duration of follow-up

Length of follow-up	**<7 years**		**7+ years**	
	Cases, n	**HR (95% CI)**	**Cases, n**	**HR (95% CI)**
Coffee intake*				
Never/monthly	23	1.0 (ref)	26	1.0 (ref)
Weekly	14	1.14 (0.72 – 1.81)	11	1.02 (0.50 – 2.07)
Daily	67	1.13 (0.80 – 1.60)	61	0.46 (0.26 – 0.81)
p for trend		0.76		0.01

*Adjusted for age (years), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), caffeine (mg/day), and total energy intake (kcal/day)

When examining covariates for confounding via stratification, the protective effect for coffee among female cohort members appeared to be confined to those women with less than secondary education (Table 49). Among these women, daily or greater coffee consumption was associated with a significant 27% reduction in gastric cancer

Table 49. Coffee intake in relation to gastric cancer risk among female members of the Singapore Chinese Health Study by education and preserved meat intake

	All women			Highest Education Achieved			
	Cases, n	HR (95% CI)		Less Than Secondary		Secondary or Higher	
Coffee intake*				Cases, n	HR (95% CI)	Cases, n	HR (95% CI)
Never/monthly	49	1.00		42	1.00	7	1.00
Weekly	25	1.27 (0.78 – 2.08)		24	1.16 (0.80 - 1.67)	1	0.54 (0.20 - 1.45)
Daily	63	0.65 (0.42 – 0.99)		55	0.72 (0.54 - 0.96)	8	1.21 (0.64 - 2.28)
2-3 cups/day	57	0.85 (0.42 – 1.69)		52	0.77 (0.52 – 1.16)	5	2.15 (0.82 – 5.59)
≥4 cups/day	8	1.03 (0.26 – 4.13)		7	0.50 (0.22 - 1.13)	1	5.23 (0.84 - 32.63)
p for trend		0.04			0.04		0.19
<Daily	74	1.00		66	1.00	8	1.00
≥Daily	128	0.60 (0.42 – 0.85)		114	0.73 (0.57 – 0.93)	14	1.35 (0.78 – 2.34)
				Quartile of Preserved Meat Intake			
				1 and 2		3 and 4	
<Daily				50	1.00	24	1.00
≥Daily				61	0.40 (0.25—0.65)	67	1.00 (0.57—1.75)

*Adjusted for age (years), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), caffeine (mg/day), and total energy intake (kcal/day)

risk, while among women with secondary or higher education, daily or greater coffee intake non-significantly increased gastric cancer risk by 35%. The interaction between education and coffee intake among women was statistically significant *(p* for interaction <0.05). When women were stratified by intake of preserved meat, the significant inverse association between daily coffee intake and gastric risk remained among those in the lowest quartiles of intake only. There remained no significant associations between coffee intake and gastric cancer risk among men after stratification by education level or preserved meat intake (data not shown).

In Table 50, we present the coffee- and caffeine-gastric cancer associations among the nested case-control dataset for which we have measured several biomarkers of *H. pylori* infection. In this subset, daily coffee drinking was inversely associated with gastric cancer risk overall, with the strongest inverse association among women. After adjustment for *H. pylori* infection status and atrophic gastritis, the associations became stronger. Among women, daily coffee intake was associated with a statistically significant 76% decrease in gastric cancer risk.

Table 50. Odds ratios (ORs) and 95% confidence intervals (95% CIs) for gastric cancer risk by coffee and caffeine intake in nested case-control study of the Singapore Chinese Health Study

	Overall			Men			Women		
Coffee Intake	**Cases/ Controls, n**	**OR (95% CI)***	**Infection-adjusted OR****	**Cases/ Controls, n**	**OR (95% CI)†**	**Infection-adjusted OR‡**	**Cases/ Controls, n**	**OR (95% CI)†**	**Infection-adjusted OR‡**
<Daily	45/107	1.00	1.00	25/72	1.00	1.00	20/35	1.00	1.00
≥Daily	88/282	0.55 (0.33—0.93)	0.51 (0.29—0.90)	60/178	0.86 (0.44—1.69)	0.74 (0.36—1.53)	28/104	0.25 (0.10—0.64)	0.24 (0.09—0.64)
Caffeine (mg/day)									
T1 (lowest)	46/119	1.00	1.00	23/69	1.00	1.00	23/50	1.00	1.00
T2	37/132	0.70 (0.42—1.19)	0.68 (0.39—1.20)	26/76	1.04 (0.54—2.02)	1.01 (0.50—2.04)	11/56	0.37 (0.16—0.87)	0.38 (0.15—0.92)
T3 (highest)	50/138	0.92 (0.55—1.53)	0.81 (0.47—1.41)	36/105	0.97 (0.51—1.84)	0.84 (0.43—1.66)	14/33	0.88 (0.37—2.10)	0.95 (0.39—2.34)
p for trend		0.39	0.42		0.97	0.82		0.06	0.08

*ORs (95% CIs) computed using conditional logistic regression adjusted for education (less than secondary/secondary or greater), smoking (never/ever), alcohol (never/ever), total energy intake (kcal/d), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and caffeine (mg/day, for coffee analyses only)

**ORs (95% CIs) computed using conditional logistic regression, adjusted for education (less than secondary/secondary or greater), smoking (never/ever), alcohol (never/ever), total energy intake (kcal/d), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), caffeine (mg/day, for coffee analyses only), *H. pylori* infection status (positive/negative), and chronic atrophic gastritis status (positive/negative)

†ORs (95% CIs) computed using unconditional logistic regression, adjusted for matching factors (i.e., age (y), dialect group (Hokkien/Cantonese), interview year (1993-1995/1996-1998), year of blood collection), and education (less than secondary/secondary or greater), smoking (never/ever), alcohol (never/ever), total energy intake (kcal/d), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and caffeine (mg/day, for coffee analyses only)

‡ORs (95% CIs) computed using unconditional logistic regression, adjusted for matching factors (i.e., age (y), dialect group (Hokkien/Cantonese), interview year (1993-1995/1996-1998), year of blood collection), and education (less than secondary/secondary or greater), smoking (never/ever), alcohol (never/ever), total energy intake (kcal/d), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), caffeine (mg/day, for coffee analyses only), *H. pylori* infection status (positive/negative), and chronic atrophic gastritis status (positive/negative)

Discussion

In the present study, novel results from analyses of coffee intake and gastric cancer risk were presented using the Singapore Chinese Health Study database, a well-established cohort with a mean follow-up of more than twelve years. Unlike other Asian populations, Singapore Chinese are frequent consumers of coffee, and the preparation techniques employed in the country may increase the exposure to chemopreventive substances in this beverage. Indeed, we reported that Singapore Chinese women who drank coffee at least once daily, compared with none or monthly intake, had a statistically significant 37% decreased risk for gastric cancer, a common cancer with relatively high mortality among this population.

Our results are similar to those of Icli et al.[311], who detected a statistically significant 50% reduction in gastric cancer risk in men and women consuming one cup of coffee per day compared to those drinking coffee less than 3 times weekly. However, the majority of research on coffee and gastric cancer has yielded null or positive associations. Botehlo et al.[369] conducted a systematic review and meta-analysis in 2006 and reported no association between coffee and gastric cancer risk (OR = 0.97; 95% CI: 0.86, 1.09) among both case-control and prospective cohort studies. Apart from one study conducted in Japan, all studies included in this meta-analysis were from Western populations; the Singapore Chinese population may differ appreciably in their relationship between coffee and gastric cancer due to coffee brewing techniques as well as higher rates of gastric cancer and *H. pylori* infection compared to Western populations. A recent examination of coffee intake and overall cancer risk in the European Prospective

Investigation into Cancer and Nutrition showed no significant protective effects[370]. Additionally, Ngoan et al.[317] did not detect an association between coffee drinking and gastric cancer risk in a prospective cohort of Japanese men and women (HR = 1.0; 95% CI: 0.9, 1.1), but relatively few subjects were daily coffee drinkers compared to Singapore Chinese (approximately 35% in the former versus approximately 87% in the latter).

In a previous prospective analysis, Larsson et al.[371] examined the association between coffee and gastric cancer risk in a cohort of Swedish women. In contrast to our findings, the authors concluded that consuming coffee at least four times daily conferred a significantly increased risk of gastric cancer compared to one cup or less daily (HR = 1.86; 95% CI: 1.07, 3.25). When the authors compared daily to less than daily coffee drinking, daily coffee consumption showed a statistically non-significant positive association (HR = 1.17; 95% CI: 0.51, 2.66). The differences in our results may be due to the much higher prevalence of female ever smokers in the Swedish versus Singapore Chinese cohort (57.8% versus 23.4% among those who drank at least four cups of coffee daily, respectively), for which the data of Larsson et al. were not adjusted. Another plausible explanation may be the differences in distribution of gastric cancer sites between Swedish and Singapore Chinese individuals; within the Singapore Chinese cohort, 14% of gastric cancers affected the gastric cardia, whereas this proportion was recently reported as 29% among the Swedish population[372]. Although there were no differences between coffee and gastric cancer risk by subsite in the present study, the higher proportions of gastric cardia cancers among Swedish people may account for the

positive association between coffee and gastric cancer risk. More research will be needed to characterize the effects of coffee on the etiology of gastric cardia versus non-cardia cancers.

More recently, Gallus et al.[373] measured a borderline significant positive trend between coffee drinking and gastric cancer risk in two Italian case-control studies with both male and female subjects (OR = 1.24; 95% CI: 0.94, 1.65; four or more cups per day versus non-drinkers; *p* for trend = 0.069). Ren et al.[374] examined the association of several beverages, including coffee, and upper gastrointestinal cancers in a large American cohort and concluded that coffee drinking significantly increased the risk of gastric cardia cancer (HR = 1.57; 95% CI: 1.03, 2.39; more than three cups daily versus less than one cup daily; *p* for trend = 0.039), while the risk of non-cardia gastric cancer was not associated with coffee consumption (HR = 1.06; 95% CI: 0.68, 1.64; more than three cups daily versus less than one cup daily; *p* for trend = 0.67). Again, the differences in etiology between gastric cardia and non-cardia cancers may explain this disparity. However, neither of these studies were conducted in an Asian population, and our results may be due to the higher prevalence of gastric cancer among such a population as well as genetic and cultural factors, such as coffee preparation techniques, that differ from the previous studies.

Our study also included a nested case-control analysis with adjustment for *H. pylori* infection and chronic atrophic gastritis, important risk factors for gastric cancer. Daily coffee drinking exerted a protective effect among women even when taking into account these risk factors, suggesting that coffee may prevent gastric cancer in a

mechanism independent of *H. pylori* infection or atrophic gastritis. However, males did not exhibit a significantly reduced risk of gastric cancer with any level of coffee consumption. It appears unlikely that the primary mechanism by which coffee reduces the risk of gastric cancer is by modulating these risk factors. Nevertheless, the antioxidants present in coffee may decrease gastritis inflammation with or without either of these conditions, thereby reducing the likelihood of mutagenesis and cancer progression[336, 366].

An additional mechanism by which coffee consumption may reduce the risk of gastric cancer is by the prevention of carcinogenesis by the nitrosamine, *N*-nitrosodimethylamine (NDMA), present in foods frequently consumed in Asian countries, such as fermented fish sauce or salted meats[268, 288, 346, 356]. Indeed, an *in vitro* study of a colon cancer cell line by Majer et al.[360] showed significant protective effects of the coffee diterpenes cafestol and kahweol against DNA damage. The diterpenes were shown to decrease the activation of procarcinogens and up-regulate the excretion of toxic metabolites by UDP-glucuronosyltransferases and glutathione. It is conceivable that such a mechanism could inhibit gastric cancer in humans[375]. However, in our study, coffee did not show a significant protective effect against gastric cancer in the highest two quartiles of preserved meat intake, suggesting that coffee drinking does not reduce the risk of gastric cancer with high nitrosamine exposure.

Nevertheless, these mechanisms do not explain the differing results between men and women in this analysis, which could not be accounted for by hormonal variables, such as parity or menopausal status. From Table 46, it is evident that men and women

have different exposures to certain covariates, including protein, dairy products, fruits, and vegetables, which are consumed more frequently among females than males, and preserved fish, shellfish, and meat, which are consumed slightly less often among females than males. Cigarette smoking is much more common among males than females. Although each of these covariates was not significant when formally analyzed for confounding, the effects of all together may be responsible for residual confounding. There is also potential for effect modification by education status, as shown in Table 49; less than secondary education may be a surrogate for lifestyle factors that would reduce the risk of gastric cancer, such as increased physical activity, access to healthcare, and consumption of fresh fruits and vegetables rather than convenience meals. An additional factor contributing to the differences in odds ratios between men and women in the nested case-control study is the relatively smaller number of women included in this analysis. The associations between coffee and gastric cancer vary between the proportional hazards regression and logistic regression analyses, and the women included in the nested case-control analyses may not be representative of the entire cohort with respect to *H. pylori* infection and chronic atrophic gastritis prevalence. This may result in selection bias that differentially affects the association between gastric cancer and coffee by gender. Finally, the lower number of female gastric cancer cases included in this study reduced the precision of relative risk measurements compared to males; the gender-specific results may be spurious and should be interpreted cautiously.

Despite the aforementioned limitations of this study, there are a number of strengths to our analyses. The prospective nature of the Singapore Chinese Health Study

allows for ascertainment of dietary and environmental exposures prior to diagnosis of cancer, thereby limiting recall bias, and the cohort has lost less than 1% of subjects to follow-up. This analysis is also unique because it is the first to examine coffee drinking habits and gastric cancer risk among the high-risk Singapore Chinese population, who consume coffee more frequently than other high-risk Asian populations. Finally, the inclusion of biomarkers benefits the analysis by providing information about the important risk factors *H. pylori* infection and chronic atrophic gastritis.

In conclusion, daily coffee consumption was associated with a significant reduction in gastric cancer risk among Singapore Chinese women. When adjustment for *H. pylori* and atrophic gastritis was included in a subset of the cohort, the negative association between coffee and gastric cancer strengthened among women. More research will be needed to elucidate the mechanisms by which coffee reduces the risk of gastric cancer as well as the differences between men and women with respect to coffee and gastric cancer risk.

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Appendix

Contamination of deconjugation enzymes from *Helix pomatia* with plant bioactive compounds 3,3′-diindolylmethane, 5-methoxypsoralen, and 8-methoxypsoralen

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The burgeoning interest in health benefits of plant bioactive compounds coupled with greater access to increasingly sensitive analytical instrumentation have increased efforts to develop biomarkers of dietary exposure to these compounds. However, despite substantial improvements in analytical instrumentation, many of the sample preparation steps preceding analyses have not similarly improved. One example of this is the enzymatic hydrolysis of the conjugated analyte of interest. *Helix pomatia* (snail) digestive juice is one typical enzyme source, which provides β-glucuronidase and sulfatase activities and returns the analyte to its unconjugated form for analysis. Nonetheless, we propose that herbivore-sourced enzyme preparations are problematic when applied to methods that quantify exposure to plant bioactives, especially when trace level analyses are required. We are in the process of validating sensitive urinary biomarkers for exposure to bioactive components found in cruciferous and apiaceous vegetables, represented by 3,3′-diindolylmethane (DIM) and the furanocoumarins 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP), respectively. It became evident that H_2O blanks treated only with the enzyme mixture and internal standard yielded detectable levels of DIM, 8-MOP, and 5-MOP. Because of the frequent experimental use of this enzyme formulation, it is imperative to inform the research community of its potential for contamination with numerous dietary components. Our objectives here are to characterize the contamination of multiple formulations of *H. pomatia* β-glucuronidase/arylsulfatase with DIM, 8-MOP, and 5-MOP and identify an alternative source of the enzyme.

To establish that *H. pomatia* β-glucuronidase/arylsulfatase (Roche cat. no. 10127698001, lot no. 70255323, Penzberg, Germany) was the source of DIM contamination, 1 mL of HPLC grade H_2O was treated with 1000, 2000, 4000, or 6000 U of the enzyme and 10 pmol of d_2-DIM (synthesized as previously described from d_2-formaldehyde, Cambridge Isotope Laboratories, Andover, MA, USA, and indole, Sigma-Aldrich, St. Louis, MO, USA [200]) as an internal standard. Additional samples were treated with 2000 U of a different lot number of the same enzyme (Roche cat. no. 10127698001, lot no. 70331220, Penzberg, Germany) or 2000 U of *E. coli* β-glucuronidase (G8295, Sigma-Aldrich) solution in phosphate buffered saline (0.26%, w/v) and internal standard. Additional replicates were treated with enzyme but no internal standard to eliminate the contribution of DIM from the deuterated internal standard. Each sample was extracted two times with an equal volume of *t*-butyl methyl ether. The extracts were evaporated to dryness and reconstituted to 20 μL with acetonitrile/10mM ammonium acetate (30/70, v/v).

To quantify furanocoumarin contamination, 3000 U of β-glucuronidase preparations from *H. pomatia* (G7017, G0751, and G1512, Sigma-Aldrich) or *Escherichia coli* (G8295, Sigma-Aldrich) were added to 750 μL LC-MS grade H_2O and 750 fmol of both internal standards (8-MOP-d_3 and 5-MOP-d_3, TLC Pharmachem, Vaughan, Ontario, Canada). Samples were extracted using Trace N SPE columns (10 mg/1 mL) on a System 96 II positive pressure manifold from SPEware (Baldwin Park, CA, USA). SPE columns were conditioned with 200 μL methanol and equilibrated with 200 μL H_2O. After the samples were loaded, columns were washed with 1 mL LC-MS

H_2O and 250 µL 85/15 (v/v) H_2O/isopropanol, followed by drying under N_2 for 8 min. Analytes were eluted with 800 µL 80/20 (v/v) hexane/ethyl acetate, and eluates were evaporated to dryness under N_2 at 40 °C. Extracts were reconstituted in 50 µL 10/90 (v/v) methanol/H_2O.

DIM quantitation was carried out by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM) on an Agilent 1100 capillary HPLC system (Agilent Technologies, Palo Alto, CA, USA) and TSQ Quantum Discovery Max instrument (Thermo Fisher Scientific, Waltham, MA, USA). MS data were acquired and processed by Xcalibur software version 1.4 (Thermo Electron, Waltham, MA, USA). Urinary DIM was quantitated in the positive ion mode with N_2 as the nebulizing and drying gas. Mass spectrometry (MS) parameters were set as follows: spray voltage, 3.2 kV; sheath gas pressure, 25; capillary temperature, 250 °C; collision energy, 17 V; scan width, 0.05 amu; Q2 gas pressure 1.0 mTorr; source CID 9 V; and tube lens offset, 104 V. Eight microliters of the sample were injected from an autosampler into the ESI source using the HPLC system equipped with a 5 µm, 150 x 0.5 mm ZorbaxSB-C18 column (Agilent Technologies, Santa Clara, CA). To optimize performance, the samples were eluted at 15 µl/min for the first 3 min then 10 µl/min with a gradient from 60% methanol in 15 mM NH_4OAc to 100% methanol in 8 min and held for additional 29 min. The mass transitions (parent to daughter) monitored were *m/z* 247→130 for DIM (retention time ranged from 16.3-17.6 min) and *m/z* 249→132 for d_2-DIM (retention time ranged from 16.1-17.5 min) as an internal standard.

Quantitation of 8-MOP and 5-MOP utilized liquid chromatography coupled with atmospheric pressure chemical ionization-tandem mass spectrometry (APCI-MS/MS). Isocratic LC separations were performed on a Restek Ultra II Aromax column (150 x 3.2 mm i.d., 3 μm, Bellefonte, PA, USA) at 600 μL/min and 50 °C, utilizing 45/15/40 methanol/acetonitrile/H_2O (v/v/v) with 0.1% acetic acid as the mobile phase. Eluent was sent to waste for the first 4 min of each run and was then introduced into the MS using a Thermo Ion Max source in APCI mode. Ionization parameters were: corona voltage, 4000 V; discharge current, 4.0 μA; vaporizer temperature, 340 °C; sheath and auxiliary gas pressure, 17 (N_2, arbitrary units); capillary temperature, 250 °C; capillary offset, 35 V; tube lens offset, 100 V; skimmer offset, -9 V; argon collision pressure, 1.6 mtorr. Retention times for 8-MOP and 8-MOP-d_3 were 5.4 min, and those for 5-MOP and 5-MOP-d_3 were 8.5 min. The nominal mass transitions *m/z* 217 → 90 (analytes) and *m/z* 220 → 90 (internal standards) were monitored in SRM mode for quantitation (collision energy = 32 eV), while *m/z* 217 → 174 and *m/z* 220 → 174 were monitored for confirmation (CE = 37 eV).

Calibration curves were constructed by plotting the peak area ratio of each analyte to its internal standard (STD:ISTD) vs. analyte concentration, and analyte concentrations within each enzyme sample were quantified by comparing the analyte:internal standard ratios from the samples to the calibration curves. The limit of quantitation (LOQ) for DIM was 132 fmol, and the LOQs of 8-MOP and 5-MOP were 113 fmol for both analytes. SAS v9.2 (SAS Institute, Inc., Cary, NC) was used for statistical analyses. The change in DIM concentration with increasing amounts of Roche *H. pomatia* β-

glucuronidase/arylsulfatase was evaluated with linear regression. The level of significance was $\alpha < 0.05$.

As shown in Figure 32, the treatment of H_2O with increasing volumes of *H. pomatia* β-glucuronidase/arylsulfatase yielded a significant positive linear association between quantity of enzyme and concentration of DIM ($R^2 = 1$, $p = 0.0013$). To verify that the contamination was not isolated to a single vial or lot of enzyme, an additional enzyme lot and an *E. coli* source of the enzyme were tested for DIM contamination and the results are shown in Table 51. Two separate lots of β-glucuronidase/arylsulfatase obtained from *H. pomatia* elicited mean DIM concentrations of 4.41 and 4.66 pmol/mL H_2O, whereas H_2O without any enzyme or with *E. coli* β-glucuronidase did not yield quantifiable concentrations of DIM.

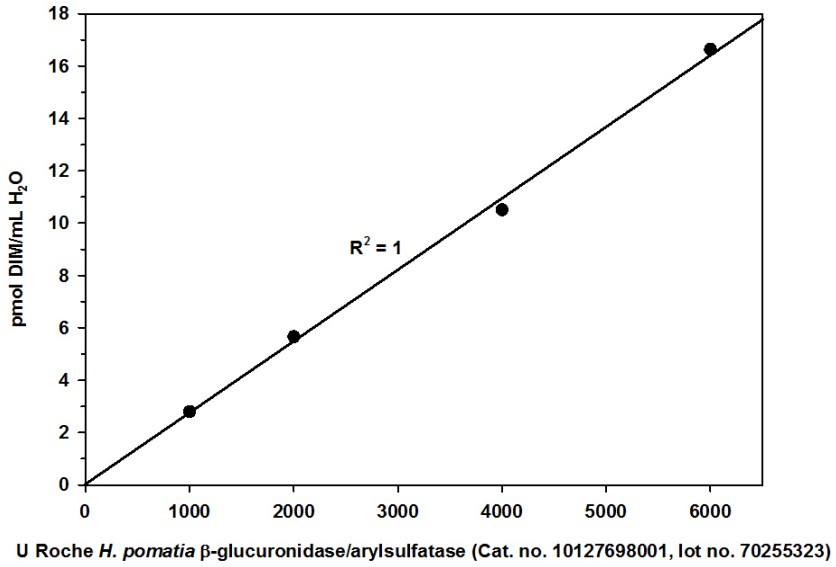

Figure 32. Change in DIM concentration with increasing amounts of *H. pomatia* β-glucuronidase $p = 0.0013$ for linear regression model

Table 51 also presents the results of 8-MOP and 5-MOP concentrations in several different preparations of *H. pomatia* β-glucuronidase/arylsulfatase and the same *E. coli* source of β-glucuronidase used to test for DIM. The mean concentrations of 8-MOP in crude and size-exclusion chromatography-purified preparations of *H. pomatia* β-glucuronidase/arylsulfatase were 3.27 and 6.03 pmol/mL H_2O, respectively. With regard to 5-MOP, the quantities detected in *H. pomatia* β-glucuronidase/arylsulfatase were lower than 8-MOP, with an average of 0.17 pmol/mL H_2O in the crude enzyme and 0.40 pmol/mL H_2O in the purified enzyme preparations. Concentrations of 8-MOP and

Table 51. Concentrations of DIM, 8-MOP, and 5-MOP detected in multiple preparations of β-glucuronidase.

β-glucuronidase preparation	**DIM (pmol/mL H_2O, mean ± standard error)[c]**	**8-MOP (pmol/mL H_2O, mean ± standard error)[d]**	**5-MOP (pmol/mL H_2O, mean ± standard error)[d]**
None[a]	<LOQ[*]	nd	nd
Roche *H. pomatia* lot #70255323[b]	4.41 ± 0.03		
Roche *H. pomatia* lot #70331220[b]	4.66 ± 0.18		
Sigma *H. pomatia* G7017 HP-2, crude solution[b]		3.27 ± 0.01	0.17 ± 0.01
Sigma *H. pomatia* G0751 H-1, partially purified powder (lot 068K38091V)		<LOQ	<LOQ
Sigma *H. pomatia* G0751 H-1, partially purified powder (lot 071M7024V)		<LOQ	<LOQ
Sigma *H. pomatia* G1512 H-5, lyophilized powder[b]		6.03 ± 0.03	0.40 ± 0.02
Sigma *E. coli* G8295[b]	nd	nd	nd

[*]Detectable levels of DIM due to contribution of d_2-DIM
[a]Average of 5 replicates
[b]Average of 2 replicates
[c]Values represent 2000 U of each enzyme
[d]Values represent 3000 U of each enzyme
LOQ = limit of quantitation (132 fmol for DIM, 113 fmol for 8-MOP and 5-MOP)

5-MOP below the LOQ were detected in two lots of a partially purified *H. pomatia* β-glucuronidase/arylsulfatase.

The results demonstrate that β-glucuronidase/arylsulfatase from *H. pomatia* is contaminated with appreciable amounts of DIM (Roche cat. no. 10127698001) as well as 8-MOP and 5-MOP (Sigma-Aldrich G7017 and G1512), thereby limiting its use in urine sample preparation for the measurement of these compounds. Additionally, 8-MOP and 5-MOP were most concentrated in a preparation purified by size-exclusion chromatography (Sigma-Aldrich G1512), demonstrating that this technique is insufficient to eliminate contaminants that may bind with high affinity to proteins in the preparation [376]. Although a partially purified preparation of β-glucuronidase/arylsulfatase from *H. pomatia* (Sigma-Aldrich G0751) did not demonstrate quantifiable contamination with 8-MOP or 5-MOP, the difficulty in predicting the distribution of contamination across samples when using a snail-sourced enzyme due to the low solubility and high protein binding of 8-MOP and 5-MOP precludes its use in these highly sensitive analyses. In contrast, recombinant β-glucuronidase produced by *E. coli* does not contain significant amounts of DIM, 8-MOP, or 5-MOP; however, it should be noted that this preparation lacks sulfatase activity. Sulfatase produced by another bacterial source, *A. aerogenes*, may be an option if this activity is also desired [377]. The identification of these compounds in β-glucuronidase/arylsulfatase from the herbivore *H. pomatia* adds to a growing list of contaminants that includes phytoestrogens and catechins [252, 377, 378]. This suggests additional plant bioactives may be present in preparations from *H. pomatia*, and underscores the risk of false positive results and misclassification of exposure status

when applying this enzyme preparation to dietary biomarker methods. We recommend cautious planning for experiments that require the use of enzyme treatment and consideration of the potential exposures of enzymes to analytes of interest and other interferences. By demonstrating that enzymes from *H. pomatia* are contaminated with plant bioactive compounds, we hope to save investigators from the time and expense associated with contamination during method development. The value of blank samples, especially those containing neither the analytical nor the internal standards, cannot be overemphasized.

Supplemental Tables from Chapter VII: Dietary fish, fat, and antioxidant consumption and the risk of gastric cancer in the Singapore Chinese Health Study

Table 52. Demographic characteristics of Singapore Chinese Healthy Study cohort members stratified by site of gastric cancer.

Characteristic	Subjects with cardia gastric cancer (n=73)	Subjects with non-cardia gastric cancer (n=345)	Subjects with unspecified gastric cancer (n=101)	Subjects without gastric cancer (n=60,802)
Male (%)	68	61	54	44
Person-years of follow-up	567	2420	631	753,440
Age at baseline, y (mean ± SD)	60.01 ± 8.39	61.29 ± 7.39	61.63 ± 7.85	56.36 ± 7.98
Hokkien dialect (%)	68	67	74	54
Body mass index, kg/m^2 (%)				
<20	10	13	12	15
20-<24	53	58	62	54
24-<28	23	21	20	24
≥28	14	8	6	7
Secondary level of education or higher (%)	19	18	17	29
Smoking status (%)				
Never	51	54	58	70
Ex-smoker	21	12	14	11
Current	29	24	28	19
Weekly or daily alcohol consumption (%)	12	16	12	12
Self-reported history of ulcer at baseline (%)	1	4	7	3
Self-reported history of diabetes at baseline (%)	11	10	5	9
Total energy intake, kcal (mean ± SD)	1617.94 ± 581.29	1539.51 ± 557.58	1467.54 ± 484.74	1556.81 ± 566.35
Total fat intake, % kcal (mean ± SD)	24.43 ± 5.58	24.47 ± 5.73	24.19 ± 5.80	25.11 ± 5.64
Total preserved fish and shellfish intake, g/kcal (mean ± SD)	1.73 ± 2.04	1.84 ± 1.79	1.62 ± 1.44	1.83 ± 2.11

Table 53. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fish intake and the risk of cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
Total fish and shellfish									
Q1	17.60 ± 6.04	22	1.00 (ref)	16.49 ± 5.49	18	1.00 (ref)	18.67 ± 6.34	4	1.00 (ref)
Q2	29.10 ± 3.25	14	0.62 (0.32—1.22)	27.61 ± 2.81	9	0.47 (0.21—1.05)	30.46 ± 3.02	5	1.37 (0.37—5.13)
Q3	39.46 ± 3.80	16	0.70 (0.37—1.34)	37.46 ± 3.26	11	0.56 (0.26—1.19)	41.06 ± 3.43	5	1.40 (0.38—5.26)
Q4	55.58 ± 13.99	21	0.90 (0.50—1.65)	53.05 ± 13.55	12	0.60 (0.29—1.26)	57.42 ± 14.01	9	2.33 (0.71—7.64)
***p* for trend**			0.48			0.22			0.51
Fresh fish and shellfish									
Q1	15.91 ± 5.73	23	1.00 (ref)	14.80 ± 5.18	19	1.00 (ref)	17.08 ± 6.02	4	1.00 (ref)
Q2	27.27 ± 3.24	13	0.55 (0.28—1.09)	25.73 ± 2.76	8	0.40 (0.18—0.92)	28.60 ± 3.00	5	1.37 (0.37—5.11)
Q3	37.54 ± 3.81	17	0.71 (0.38—1.34)	35.45 ± 3.23	11	0.53 (0.25—1.13)	39.19 ± 3.41	6	1.68 (0.47—5.97)
Q4	53.50 ± 14.01	20	0.82 (0.45—1.51)	50.85 ± 13.55	12	0.58 (0.28—1.21)	55.52 ± 14.02	8	2.03 (0.61—6.80)
***p* for trend**			0.37			0.11			0.70
Preserved fish and shellfish									
Q1	0.16 ± 0.13	23	1.00 (ref)	0.19 ± 0.15	17	1.00 (ref)	0.15 ± 0.12	6	1.00 (ref)
Q2	0.80 ± 0.25	20	0.85	0.88 ± 0.24	14	0.77	0.73 ± 0.23	6	1.11

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
			(0.47—1.56)			(0.38—1.58)			(0.36—3.45)
Q3	1.79 ± 0.37	11	0.46 (0.22—0.95)	1.85 ± 0.36	8	0.42 (0.18—0.98)	1.73 ± 0.36	3	0.60 (0.15—2.43)
Q4	3.75 ± 2.57	19	0.79 (0.43—1.47)	3.83 ± 2.59	11	0.56 (0.26—1.20)	3.69 ± 2.56	8	1.69 (0.57—4.97)
***p* for trend**			0.21			0.19			0.47
Fish, fresh and preserved									
Q1	15.03 ± 5.50	21	1.00 (ref)	13.95 ± 4.96	18	1.00 (ref)	16.11 ± 5.78	3	1.00 (ref)
Q2	25.93 ± 3.13	17	0.79 (0.42—1.50)	24.44 ± 2.69	10	0.52 (0.24—1.13)	27.21 ± 2.86	7	2.55 (0.66—9.89)
Q3	35.90 ± 3.74	16	0.72 (0.38—1.39)	33.82 ± 3.13	12	0.60 (0.29—1.25)	37.57 ± 3.37	4	1.48 (0.33—6.66)
Q4	51.73 ± 13.95	19	0.84 (0.45—1.57)	53.06 ± 13.52	10	0.49 (0.23—1.08)	53.69 ± 13.93	9	3.04 (0.82—11.33)
***p* for trend**			0.79			0.21			0.32
Shellfish, fresh and preserved									
Q1	0.69 ± 0.41	18	1.00 (ref)	0.81 ± 0.43	13	1.00 (ref)	0.61 ± 0.38	5	1.00 (ref)
Q2	1.92 ± 0.36	18	1.06 (0.55—2.05)	2.03 ± 0.34	12	0.94 (0.43—2.06)	1.82 ± 0.35	6	1.46 (0.44—4.83)
Q3	3.26 ± 0.50	17	1.05 (0.54—2.05)	3.34 ± 0.47	13	1.05 (0.48—2.28)	3.19 ± 0.51	4	1.05 (0.28—3.96)
Q4	6.06 ± 3.19	20	1.21 (0.63—2.32)	6.02 ± 2.87	12	0.93 (0.42—2.07)	6.11 ± 3.42	8	2.14 (0.68—6.71)
***p* for trend**			0.94			0.99			0.52

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 54. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fat intakes and the risk of cardia gastric cancer in the Singapore Chinese Health Study..

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Total Fat									
Q1	18.57 ± 2.81	19	1.00 (ref)	17.71 ± 2.71	10	1.00 (ref)	19.29 ± 2.71	9	1.00 (ref)
Q2	23.33 ± 1.26	17	0.95 (0.50—1.84)	22.62 ± 1.14	12	1.25 (0.54—2.90)	23.88 ± 1.07	5	0.64 (0.21—1.93)
Q3	26.93 ± 1.23	23	1.33 (0.72—2.46)	26.27 ± 1.10	16	1.67 (0.75—3.70)	27.44 ± 1.09	7	1.02 (0.37—2.82)
Q4	31.60 ± 2.74	14	0.85 (0.42—1.74)	31.05 ± 2.76	12	1.25 (0.53—2.98)	32.02 ± 2.65	2	0.34 (0.07—1.66)
***p* for trend**			0.56			0.63			0.48
Saturated Fat									
Q1	5.92 ± 1.01	17	1.00 (ref)	5.76 ± 0.99	9	1.00 (ref)	6.06 ± 1.01	8	1.00 (ref)
Q2	7.93 ± 0.51	25	1.57 (0.84—2.91)	7.77 ± 0.49	18	2.09 (0.94—4.67)	8.16 ± 0.49	7	0.97 (0.35—2.70)
Q3	9.61 ± 0.54	15	0.96 (0.48—1.94)	9.41 ± 0.52	10	1.16 (0.47—2.88)	9.75 ± 0.52	5	0.74 (0.24—2.32)
Q4	11.85 ± 1.33	16	1.05 (0.52—2.12)	11.67 ± 1.34	13	1.51 (0.63—3.62)	12.01 ± 1.31	3	0.49 (0.13—1.93)
***p* for trend**			0.35			0.25			0.74
MUFA									
Q1	6.10 ± 0.96	13	1.00 (ref)	5.87 ± 0.93	7	1.00 (ref)	6.30 ± 0.93	6	1.00 (ref)
Q2	7.79 ± 0.43	24	1.97	7.59 ± 0.40	15	2.20	7.94 ± 0.39	9	1.76

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
			(1.00—3.87)			(0.90—5.41)			(0.62—4.97)
Q3	9.11 ± 0.43	17	1.44 (0.70—2.98)	8.94 ± 0.41	12	1.79 (0.70—4.57)	9.25 ± 0.40	5	1.10 (0.33—3.67)
Q4	10.88 ± 1.09	19	1.69 (0.82—3.48)	10.73 ± 1.10	16	2.41 (0.97—5.97)	10.99 ± 1.07	3	0.76 (0.18—3.17)
***p* for trend**			0.25			0.26			0.55
PUFA									
Q1	3.24 ± 0.55	28	1.00 (ref)	3.09 ± 0.52	16	1.00 (ref)	3.38 ± 0.54	12	1.00 (ref)
Q2	4.26 ± 0.31	13	0.49 (0.25—0.95)	4.08 ± 0.25	11	0.70 (0.33—1.52)	4.42 ± 0.27	2	0.20 (0.04—0.89)
Q3	5.27 ± 0.43	12	0.47 (0.24—0.93)	5.03 ± 0.34	8	0.51 (0.21—1.20)	5.45 ± 0.39	4	0.46 (0.14—1.44)
Q4	7.30 ± 1.48	20	0.81 (0.45—1.46)	6.90 ± 1.38	15	0.97 (0.47—2.01)	7.62 ± 1.49	5	0.60 (0.21—1.77)
***p* for trend**			0.06			0.37			0.15
Total n-3									
Q1	0.36 ± 0.055	22	1.00 (ref)	0.34 ± 0.051	15	1.00 (ref)	0.38 ± 0.054	7	1.00 (ref)
Q2	0.45 ± 0.030	23	1.06 (0.59—1.90)	0.43 ± 0.022	17	1.10 (0.55—2.21)	0.47 ± 0.023	6	1.00 (0.33—3.00)
Q3	0.53 ± 0.033	14	0.64 (0.32—1.25)	0.51 ± 0.025	11	0.68 (0.31—1.48)	0.55 ± 0.026	3	0.53 (0.14—2.07)
Q4	0.67 ± 0.18	14	0.64 (0.33—1.26)	0.64 ± 0.16	7	0.43 (0.17—1.06)	0.69 ± 0.19	7	1.29 (0.45—3.74)
***p* for trend**			0.27			0.15			0.64
Marine n-3									
Q1	0.092 ± 0.032	23	1.00 (ref)	0.087 ± 0.029	18	1.00 (ref)	0.097 ± 0.033	5	1.00 (ref)
Q2	0.15 ± 0.017	14	0.60	0.15 ±	10	0.53	0.16 ±	4	0.89

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
			(0.31—1.17)	0.015		(0.24—1.14)	0.016		(0.24—3.31)
Q3	0.21 ± 0.019	16	0.67 (0.36—1.28)	0.20 ± 0.017	12	0.61 (0.30—1.28)	0.21 ± 0.018	4	0.91 (0.24—3.40)
Q4	0.29 ± 0.072	20	0.82 (0.45—1.51)	0.28 ± 0.071	10	0.50 (0.23—1.09)	0.30 ± 0.072	10	2.12 (0.72—6.26)
***p* for trend**			0.43			0.22			0.29
Non-marine n-3									
Q1	0.21 ± 0.033	25	1.00 (ref)	0.21 ± 0.030	12	1.00 (ref)	0.22 ± 0.032	13	1.00 (ref)
Q2	0.28 ± 0.019	21	0.87 (0.48—1.56)	0.26 ± 0.015	18	1.49 (0.71—3.11)	0.29 ± 0.015	3	0.27 (0.08—0.97)
Q3	0.33 ± 0.021	18	0.75 (0.41—1.39)	0.31 ± 0.017	13	1.07 (0.48—2.38)	0.34 ± 0.018	5	0.48 (0.17—1.39)
Q4	0.42 ± 0.18	9	0.38 (0.18—0.82)	0.40 ± 0.16	7	0.57 (0.22—1.47)	0.44 ± 0.19	2	0.21 (0.05—0.93)
***p* for trend**			0.10			0.19			0.06
Total n-6									
Q1	2.82 ± 0.49	27	1.00 (ref)	2.69 ± 0.46	15	1.00 (ref)	2.95 ± 0.48	12	1.00 (ref)
Q2	3.75 ± 0.29	15	0.59 (0.31—1.10)	3.59 ± 0.23	12	0.82 (0.38—1.75)	3.89 ± 0.25	3	0.30 (0.08—1.07)
Q3	4.70 ± 0.41	11	0.45 (0.22—0.91)	4.47 ± 0.32	8	0.54 (0.23—1.30)	4.87 ± 0.38	3	0.34 (0.09—1.22)
Q4	6.64 ± 1.40	20	0.84 (0.46—1.52)	6.26 ± 1.30	15	1.04 (0.50—2.18)	6.95 ± 1.40	5	0.60 (0.20—1.75)
***p* for trend**			0.10			0.46			0.16
Total animal fat									
Q1	4.57 ± 1.27	21	1.00 (ref)	4.82 ± 1.30	13	1.00 (ref)	4.41 ± 1.21	8	1.00 (ref)
Q2	7.14 ± 0.70	17	0.86 (0.45—1.63)	7.49 ± 0.64	13	1.04 (0.48—2.25)	6.87 ± 0.61	4	0.59 (0.18—1.98)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Q3	9.31 ± 0.78	17	0.90 (0.47—1.72)	9.70 ± 0.71	8	0.65 (0.27—1.58)	8.99 ± 0.66	9	1.53 (0.58—4.06)
Q4	12.40 ± 2.23	18	0.99 (0.52—1.90)	12.93 ± 2.23	16	1.31 (0.61—2.80)	11.93 ± 2.12	2	0.38 (0.08—1.85)
***p* for trend**			0.96			0.46			0.21
Total red meat fat									
Q1	1.08 ± 0.49	22	1.00 (ref)	1.25 ± 0.51	12	1.00 (ref)	0.96 ± 0.44	10	1.00 (ref)
Q2	2.20 ± 0.34	15	0.70 (0.36—1.35)	2.40 ± 0.30	11	0.92 (0.40—2.08)	2.05 ± 0.29	4	0.45 (0.14—1.43)
Q3	3.28 ± 0.43	18	0.85 (0.45—1.59)	3.52 ± 0.38	15	1.26 (0.58—2.70)	3.08 ± 0.36	3	0.37 (0.10—1.34)
Q4	5.08 ± 1.58	18	0.85 (0.45—1.61)	5.40 ± 1.63	12	0.96 (0.42—2.19)	4.81 ± 1.47	6	0.79 (0.28—2.24)
***p* for trend**			0.76			0.85			0.34
Total plant fat									
Q1	11.65 ± 2.02	22	1.00 (ref)	10.74 ± 1.76	17	1.00 (ref)	12.61 ± 1.85	5	1.00 (ref)
Q2	15.06 ± 1.23	18	0.84 (0.45—1.56)	14.03 ± 0.80	7	0.41 (0.17—0.99)	15.92 ± 0.79	11	2.47 (0.85—7.12)
Q3	17.80 ± 1.28	15	0.72 (0.37—1.39)	16.71 ± 0.83	10	0.59 (0.27—1.29)	18.63 ± 0.86	5	1.24 (0.36—4.35)
Q4	21.74 ± 2.92	18	0.88 (0.47—1.66)	20.48 ± 2.72	16	0.94 (0.46—1.88)	22.52 ± 2.76	2	0.53 (0.10—2.77)
***p* for trend**			0.80			0.15			0.12
Total cholesterol									
Q1	62.53 ± 15.42	22	1.00 (ref)	62.88 ± 15.37	15	1.00 (ref)	62.30 ± 15.45	7	1.00 (ref)
Q2	92.04 ± 7.33	13	0.63	93.16 ±	9	0.61	90.98 ±	4	0.69

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
			(0.32—1.26)	7.37		(0.27—1.41)	7.16		(0.20—2.38)
Q3	116.95 ± 8.35	20	1.01 (0.55—1.86)	118.83 ± 8.56	12	0.83 (0.39—1.79)	115.49 ± 7.85	8	1.56 (0.56—4.39)
Q4	156.29 ± 42.14	16	0.93 (0.49—1.76)	161.28 ± 47.29	14	0.98 (0.47—2.07)	152.25 ± 36.47	4	0.82 (0.23—2.87)
***p* for trend**			0.54			0.65			0.53

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 55. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of antioxidant intake and the risk of cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
Total selenium (μg/kcal)									
Q1	53.81 ± 5.07	20	1.00 (ref)	53.76 ± 4.85	15	1.00 (ref)	53.83 ± 5.25	5	1.00 (ref)
Q2	61.56 ± 1.88	17	0.82 (0.43—1.57)	61.09 ± 1.75	12	0.76 (0.36—1.63)	61.96 ± 1.90	5	1.01 (0.29—3.50)
Q3	67.82 ± 2.11	17	0.80 (0.42—1.53)	67.11 ± 1.94	12	0.74 (0.34—1.58)	68.37 ± 2.07	5	1.00 (0.29—3.48)
Q4	77.31 ± 8.75	19	0.86 (0.46—1.63)	76.03 ± 7.95	11	0.66 (0.30—1.46)	78.19 ± 9.23	8	1.47 (0.47—4.54)
***p* for trend**			0.90			0.75			0.87
Total									

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
carotenoids (μg/kcal)									
Q1	1771.00 ± 499.15	28	1.00 (ref)	1569.97 ± 430.42	20	1.00 (ref)	1980.15 ± 476.48	8	1.00 (ref)
Q2	2795.98 ± 376.33	16	0.60 (0.33—1.12)	2524.97 ± 256.31	10	0.51 (0.24—1.09)	3045.52 ± 283.47	6	0.85 (0.29—2.46)
Q3	3844.34 ± 487.95	15	0.60 (0.32—1.13)	3486.02 ± 340.25	11	0.60 (0.28—1.26)	4125.13 ± 385.84	4	0.60 (0.18—2.00)
Q4	5811.57 ± 1948.95	14	0.60 (0.31—1.16)	5265.59 ± 1754.35	9	0.53 (0.24—1.18)	6201.37 ± 1980.48	5	0.76 (0.24—2.40)
***p* for trend**			0.23			0.22			0.86
α-carotene (μg/kcal)									
Q1	29.86 ± 20.52	22	1.00 (ref)	20.07 ± 13.44	15	1.00 (ref)	42.03 ± 21.37	7	1.00 (ref)
Q2	85.81 ± 22.09	19	0.86 (0.46—1.59)	68.89 ± 13.81	15	0.95 (0.46—1.95)	100.43 ± 16.50	4	0.61 (0.18—2.10)
Q3	153.81 ± 36.78	17	0.78 (0.41—1.48)	126.96 ± 23.53	10	0.64 (0.29—1.44)	176.40 ± 30.25	7	1.12 (0.39—3.20)
Q4	332.95 ± 224.87	15	0.72 (0.37—1.40)	278.20 ± 187.03	10	0.69 (0.31—1.54)	375.39 ± 239.39	5	0.80 (0.25—2.55)
***p* for trend**			0.78			0.62			0.79
β-carotene (μg/kcal)									
Q1	635.74 ± 201.97	24	1.00 (ref)	538.81 ± 160.12	18	1.00 (ref)	747.12 ± 186.11	6	1.00 (ref)
Q2	1038.41 ± 171.11	20	0.87 (0.48—1.58)	898.74 ± 97.04	12	0.68 (0.33—1.42)	1164.68 ± 116.11	8	1.43 (0.50—4.14)
Q3	1465.99 ± 232.05	18	0.82 (0.44—1.51)	1275.45 ± 132.95	13	0.77 (0.38—1.59)	1626.14 ± 164.74	5	0.91 (0.28—3.02)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q4	2299.51 ± 880.12	11	0.53 (0.26—1.08)	1975.03 ± 741.98	7	0.44 (0.18—1.07)	2521.20 ± 892.44	4	0.75 (0.21—2.71)
***p* for trend**			0.37			0.32			0.73
β-cryptoxanthin (μg/kcal)									
Q1	12.06 ± 14.35	22	1.00 (ref)	13.28 ± 14.40	10	1.00 (ref)	10.73 ± 14.30	12	1.00 (ref)
Q2	71.46 ± 17.14	20	1.01 (0.55—1.86)	69.13 ± 15.88	15	1.56 (0.70—3.50)	73.50 ± 17.87	5	0.52 (0.18—1.51)
Q3	142.32 ± 28.18	19	1.00 (0.54—1.87)	136.38 ± 26.63	14	1.50 (0.66—3.41)	147.06 ± 28.41	5	0.56 (0.19—1.65)
Q4	323.55 ± 283.03	12	0.62 (0.30—1.28)	309.38 ± 254.88	11	1.16 (0.48—2.48)	333.47 ± 302.25	1	0.11 (0.01—0.89)
***p* for trend**			0.53			0.66			0.15
Lycopene (μg/kcal)									
Q1	90.53 ± 61.09	21	1.00 (ref)	105.40 ± 65.89	13	1.00 (ref)	80.26 ± 54.58	8	1.00 (ref)
Q2	312.87 ± 79.75	14	0.73 (0.37—1.43)	345.30 ± 78.00	10	0.79 (0.34—1.80)	287.52 ± 72.27	4	0.65 (0.19—2.18)
Q3	627.01 ± 119.05	23	1.27 (0.69—2.31)	657.09 ± 111.43	17	1.41 (0.68—2.93)	603.63 ± 119.41	6	1.10 (0.37—3.25)
Q4	1311.77 ± 1210.93	15	0.93 (0.47—1.83)	1396.34 ± 1196.84	10	0.94 (0.40—2.18)	1245.72 ± 1219.55	5	0.96 (0.30—3.05)
***p* for trend**			0.42			0.48			0.87
Lutein (μg/kcal)									
Q1	597.01 ± 173.40	24	1.00 (ref)	520.16 ± 139.35	19	1.00 (ref)	688.74 ± 163.54	5	1.00 (ref)
Q2	939.52 ± 143.27	19	0.84 (0.46—1.54)	825.32 ± 81.85	11	0.61 (0.29—1.28)	1045.90 ± 97.82	8	1.76 (0.57—5.39)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q3	1296.07 ± 191.42	15	0.69 (0.36—1.32)	1132.96 ± 110.31	11	0.64 (0.30—1.35)	1427.47 ± 134.42	4	0.87 (0.23—3.27)
Q4	1949.59 ± 639.28	15	0.70 (0.37—1.35)	1684.55 ± 518.06	9	0.54 (0.24—1.20)	2128.98	6	1.23 (0.37—4.11)
***p* for trend**			0.63			0.36			0.64
Total vitamin C (mg/kcal)									
Q1	21.66 ± 6.76	25	1.00 (ref)	19.99 ± 6.50	14	1.00 (ref)	23.02 ± 6.68	11	1.00 (ref)
Q2	39.63 ± 5.71	14	0.61 (0.31—1.17)	37.35 ± 4.99	11	0.80 (0.36—1.76)	41.60 ± 5.50	3	0.33 (0.09—1.20)
Q3	60.24 ± 8.33	22	1.03 (0.57—1.85)	56.55 ± 6.84	16	1.25 (0.60—2.60)	63.40 ± 8.09	6	0.72 (0.26—2.02)
Q4	102.73 ± 151.60	12	0.59 (0.29—1.19)	94.37 ± 127.18	9	0.74 (0.31—1.76)	108.74 ± 167.54	3	0.37 (0.10—1.39)
***p* for trend**			0.20			0.55			0.25
Total vitamin E (mg α-tocopherol equivalents/kcal)									
Q1	2.59 ± 0.47	25	1.00 (ref)	2.38 ± 0.40	17	1.00 (ref)	2.84 ± 0.43	8	1.00 (ref)
Q2	3.41 ± 0.31	16	0.67 (0.36—1.26)	3.14 ± 0.19	9	0.54 (0.24—1.21)	3.64 ± 0.20	7	1.01 (0.36—2.81)
Q3	4.10 ± 0.34	14	0.62 (0.32—1.20)	3.80 ± 0.21	11	0.68 (0.32—1.46)	4.33 ± 0.23	3	0.48 (0.13—1.84)
Q4	5.25 ± 64.00	18	0.82 (0.44—1.53)	4.85 ± 54.66	13	0.81 (0.39—1.69)	5.49 ± 70.52	5	0.86 (0.27—2.72)
***p* for trend**			0.45			0.48			0.72
Total soy isoflavones									

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
(mg/kcal)									
Q1	3.63 ± 1.65	22	1.00 (ref)	3.25 ± 1.45	14	1.00 (ref)	4.00 ± 1.72	8	1.00 (ref)
Q2	7.74 ± 1.35	21	0.98 (0.54—1.78)	7.02 ± 1.07	13	0.90 (0.42—1.91)	8.37 ± 1.24	8	1.19 (0.44—3.20)
Q3	12.24 ± 1.87	17	0.80 (0.42—1.51)	11.12 ± 1.46	15	1.01 (0.48—2.11)	13.15 ± 1.66	2	0.33 (0.07—1.56)
Q4	20.72 ± 9.85	13	0.61 (0.31—1.22)	19.12 ± 8.76	8	0.53 (0.22—1.28)	22.00 ± 10.39	5	0.85 (0.27—2.66)
***p* for trend**			0.49			0.47			0.43
Genistein (mg/kcal)									
Q1	1.70 ± 0.76	22	1.00 (ref)	1.52 ± 0.67	15	1.00 (ref)	1.88 ± 0.80	7	1.00 (ref)
Q2	3.58 ± 0.62	22	1.03 (0.57—1.86)	3.24 ± 0.49	13	0.84 (0.40—1.77)	3.87 ± 0.56	9	1.55 (0.57—4.20)
Q3	5.63 ± 0.85	15	0.70 (0.36—1.36)	5.10 ± 0.66	13	0.81 (0.39—1.72)	6.04 ± 0.75	2	0.38 (0.08—1.83)
Q4	9.46 ± 4.37	14	0.66 (0.34—1.30)	8.72 ± 3.90	9	0.56 (0.24—1.29)	10.04 ± 4.59	5	0.98 (0.31—3.16)
***p* for trend**			0.43			0.60			0.32
Daidzein (mg/kcal)									
Q1	1.69 ± 0.78	20	1.00 (ref)	1.52 ± 0.69	12	1.00 (ref)	1.86 ± 0.81	8	1.00 (ref)
Q2	3.65 ± 0.64	23	1.18 (0.65—2.15)	3.31 ± 0.52	15	1.21 (0.57—2.59)	3.96 ± 0.59	8	1.20 (0.45—3.22)
Q3	5.81 ± 0.90	17	0.89 (0.46—1.70)	5.28 ± 0.70	15	1.19 (0.55—2.57)	6.24 ± 0.80	2	0.33 (0.07—1.56)
Q4	9.92 ± 4.84	13	0.67 (0.33—1.34)	9.14 ± 4.29	8	0.62 (0.25—1.54)	10.55 ± 5.12	5	0.86 (0.28—2.67)
***p* for trend**			0.44			0.44			0.43
Glycitein									

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
(mg/kcal)									
Q1	0.24 ± 0.11	21	1.00 (ref)	0.21 ± 0.096	13	1.00 (ref)	0.26 ± 0.11	8	1.00 (ref)
Q2	0.51 ± 0.087	21	1.03 (0.56—1.88)	0.46 ± 0.070	13	0.97 (0.45—2.10)	0.55 ± 0.080	8	1.19 (0.44—3.20)
Q3	0.80 ± 0.12	18	0.89 (0.47—1.68)	0.73 ± 0.096	16	1.17 (0.56—2.45)	0.86 ± 0.11	2	0.33 (0.07—1.56)
Q4	1.36 ± 0.65	13	0.64 (0.32—1.29)	1.25 ± 0.58	8	0.57 (0.24—1.40)	1.44 ± 0.69	5	0.85 (0.27—2.66)
***p* for trend**			0.56			0.43			0.43
Total isothiocyanate (μmol/kcal)									
Q1	2.39 ± 0.84	21	1.00 (ref)	2.06 ± 0.71	13	1.00 (ref)	2.71 ± 0.83	8	1.00 (ref)
Q2	4.26 ± 0.68	15	0.75 (0.39—1.46)	3.79 ± 0.48	11	0.88 (0.39—1.96)	4.68 ± 0.54	4	0.55 (0.17—1.85)
Q3	6.25 ± 0.92	25	1.31 (0.73—2.35)	5.59 ± 0.66	18	1.52 (0.74—3.11)	6.77 ± 0.75	7	0.99 (0.36—2.76)
Q4	10.08 ± 4.36	12	0.64 (0.31—1.31)	9.08 ± 3.79	8	0.69 (0.28—1.67)	10.81 ± 4.58	4	0.56 (0.17—1.88)
***p* for trend**			0.15			0.23			0.62

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 56. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for tea and coffee intakes and the risk of cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Black tea (cups/month)									
Q1	0.00	43	1.00 (ref)	0.00	26	1.00 (ref)	0.00	17	1.00 (ref)
Q2	2.00 ± 1.14	13	1.52 (0.82—2.84)	2.00 ± 1.14	9	1.53 (0.72—3.28)	2.00 ± 1.13	4	1.56 (0.52—4.69)
Q3	10.70 ± 4.48	7	0.87 0.39—1.94)	10.70 ± 4.61	6	0.96 (0.39—2.33)	10.70 ± 4.30	1	0.62 (0.08—4.72)
Q4	30.00 ± 26.28	10	1.17 (0.58—2.36)	30.00 ± 27.73	9	1.27 (0.59—2.74)	30.00 ± 23.36	1	0.75 (0.10—5.70)
***p* for trend**			0.54			0.68			0.79
Green tea (cups/month)									
Q1	0.00	38	1.00 (ref)	0.00	25	1.00 (ref)	0.00	13	1.00 (ref)
Q2	2.00 ± 1.11	20	1.77 (1.03—3.06)	2.00 ± 1.11	13	1.68 (0.86—3.30)	2.00 ± 1.10	7	2.02 (0.80—5.09)
Q3	10.70 ± 4.28	5	0.68 (0.27—1.75)	10.70 ± 4.44	5	0.87 (0.33—2.27)	10.70 ± 4.09	0	0.00 (n/a)
Q4	30.00 ± 40.71	10	1.09 (0.53—2.21)	75.00 ± 42.47	7	0.93 (0.40—2.19)	30.00 ± 38.12	3	1.62 (0.45—5.82)
***p* for trend**			0.12			0.39			0.51
Any tea (cups/month)									
Q1	0.00	29	1.00 (ref)	0.00	17	1.00 (ref)	0.00	12	1.00 (ref)
Q2	4.00 ± 1.83	19	1.32 (0.74—2.37)	4.00 ± 1.87	12	1.28 (0.61—2.69)	4.00 ± 1.79	7	1.43 (0.56—3.67)
Q3	10.70 ± 4.96	8	0.64 (0.29—1.42)	12.70 ± 5.09	7	0.75 (0.31—1.81)	10.70 ± 4.80	1	0.36 (0.05—2.76)
Q4	40.70 ± 39.87	17	0.90 (0.49—1.67)	40.70 ± 41.56	14	0.91 (0.45—1.87)	34.30 ± 37.07	3	0.89 (0.25—3.20)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
***p* for trend**			0.37			0.69			0.58
Coffee (cups/week)									
Never	0.00	15	1.00 (ref)	0.00	8	1.00 (ref)	0.00	7	1.00 (ref)
Monthly	0.46 ± 0.15	1	0.64 (0.08—4.81)	0.46 ± 0.15	1	1.18 (0.15—9.49)	0.46 ± 0.15	0	0.00 (n/a)
Weekly	2.50 ± 1.46	10	1.35 (0.60—3.01)	2.50 ± 1.45	7	1.74 (0.63—4.83)	2.50 ± 1.47	3	0.94 (0.24—3.63)
1 cup/day	7.02 ± 0.79	22	0.77 (0.40—1.48)	7.02 ± 0.83	12	0.84 (0.34—2.05)	7.02 ± 0.77	10	0.65 (0.25—1.71)
2-3 cups/day	17.56 ± 1.96	23	0.83 (0.43—1.60)	17.56 ± 1.97	20	1.19 (0.52—2.74)	17.56 ± 1.96	3	0.32 (0.08—1.26)
≥4 cups/day	31.60 ± 5.19	2	0.47 (0.11—2.09)	31.60 ± 4.68	2	0.70 (0.15—3.35)	31.60 ± 5.95	0	0.00 (n/a)
***p* for trend**			0.64			0.70			0.70

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 57. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fish intake and the risk of non-cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
Total fish and shellfish									
Q1	17.60 ± 6.04	94	1.00 (ref)	16.49 ± 5.49	59	1.00 (ref)	18.67 ± 6.34	35	1.00 (ref)
Q2	29.10 ± 3.25	76	0.82 (0.61—1.11)	27.61 ± 2.81	43	0.71 (0.48—1.05)	30.46 ± 3.02	33	1.00 (0.62—1.61)
Q3	39.46 ± 3.80	87	0.93 (0.69—1.25)	37.46 ± 3.26	55	0.90 (0.63—1.31)	41.06 ± 3.43	32	0.95 (0.59—1.54)
Q4	55.58 ± 13.99	88	0.92 (0.68—1.23)	53.05 ± 13.55	55	0.88 (0.61—1.28)	57.42 ± 14.01	33	0.96 (0.59—1.55)
***p* for trend**			0.64			0.39			1.00
Fresh fish and shellfish									
Q1	15.91 ± 5.73	90	1.00 (ref)	14.80 ± 5.18	56	1.00 (ref)	17.08 ± 6.02	34	1.00 (ref)
Q2	27.27 ± 3.24	81	0.91 (0.67—1.23)	25.73 ± 2.76	47	0.81 (0.55—1.20)	28.60 ± 3.00	34	1.05 (0.66—1.70)
Q3	37.54 ± 3.81	83	0.92 (0.68—1.24)	35.45 ± 3.23	51	0.88 (0.60—1.28)	39.19 ± 3.41	32	0.98 (0.61—1.60)
Q4	53.50 ± 14.01	91	0.99 (0.74—1.32)	50.85 ± 13.55	58	0.98 (0.68—1.42)	55.52 ± 14.02	33	0.98 (0.60—1.59)
***p* for trend**			0.90			0.69			0.99
Preserved fish and shellfish									
Q1	0.16 ± 0.13	89	1.00 (ref)	0.19 ± 0.15	53	1.00 (ref)	0.15 ± 0.12	36	1.00 (ref)
Q2	0.80 ± 0.25	77	0.87 (0.64—1.18)	0.88 ± 0.24	48	0.91 (0.62—1.35)	0.73 ± 0.23	29	0.82 (0.50—1.34)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Q3	1.79 ± 0.37	86	0.97 (0.72—1.31)	1.85 ± 0.36	48	0.90 (0.60—1.33)	1.73 ± 0.36	38	1.10 (0.69—1.74)
Q4	3.75 ± 2.57	93	1.07 (0.79—1.43)	3.83 ± 2.59	63	1.19 (0.82—1.73)	3.69 ± 2.56	30	0.88 (0.54—1.44)
***p* for trend**			0.61			0.41			0.64
Fish, fresh and preserved									
Q1	15.03 ± 5.50	89	1.00 (ref)	13.95 ± 4.96	53	1.00 (ref)	16.11 ± 5.78	36	1.00 (ref)
Q2	25.93 ± 3.13	87	0.98 (0.73—1.32)	24.44 ± 2.69	56	1.01 (0.70—1.48)	27.21 ± 2.86	31	0.90 (0.56—1.46)
Q3	35.90 ± 3.74	72	0.79 (0.58—1.08)	33.82 ± 3.13	44	0.78 (0.52—1.17)	37.57 ± 3.37	28	0.80 (0.49—1.31)
Q4	51.73 ± 13.95	97	1.04 (0.78—1.39)	53.06 ± 13.52	59	1.03 (0.71—1.49)	53.69 ± 13.93	38	1.04 (0.66—1.65)
***p* for trend**			0.34			0.49			0.72
Shellfish, fresh and preserved									
Q1	0.69 ± 0.41	104	1.00 (ref)	0.81 ± 0.43	54	1.00 (ref)	0.61 ± 0.38	50	1.00 (ref)
Q2	1.92 ± 0.36	86	0.92 (0.69—1.22)	2.03 ± 0.34	51	1.04 (0.71—1.52)	1.82 ± 0.35	35	0.79 (0.51—1.22)
Q3	3.26 ± 0.50	72	0.81 (0.60—1.10)	3.34 ± 0.47	48	1.03 (0.70—1.53)	3.19 ± 0.51	24	0.57 (0.35—0.93)
Q4	6.06 ± 3.19	83	0.96 (0.71—1.29)	6.02 ± 2.87	59	1.28 (0.88—1.86)	6.11 ± 3.42	24	0.59 (0.36—0.97)
***p* for trend**			0.59			0.56			0.07

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 58. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fat intakes and the risk of non-cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
Total Fat									
Q1	18.57 ± 2.81	91	1.00 (ref)	17.71 ± 2.71	56	1.00 (ref)	19.29 ± 2.71	35	1.00 (ref)
Q2	23.33 ± 1.26	93	1.13 (0.84—1.51)	22.62 ± 1.14	57	1.09 (0.75—1.57)	23.88 ± 1.07	36	1.18 (0.74—1.88)
Q3	26.93 ± 1.23	89	1.17 (0.87—1.57)	26.27 ± 1.10	54	1.10 (0.75—1.60)	27.44 ± 1.09	35	1.29 (0.80—2.07)
Q4	31.60 ± 2.74	72	1.07 (0.78—1.47)	31.05 ± 2.76	45	1.02 (0.68—1.52)	32.02 ± 2.65	27	1.14 (0.68—1.93)
***p* for trend**			0.74			0.95			0.78
Saturated Fat									
Q1	5.92 ± 1.01	88	1.00 (ref)	5.76 ± 0.99	56	1.00 (ref)	6.06 ± 1.01	32	1.00 (ref)
Q2	7.93 ± 0.51	79	1.01 (0.74—1.36)	7.77 ± 0.49	50	1.00 (0.68—1.46)	8.16 ± 0.49	29	1.03 (0.62—1.70)
Q3	9.61 ± 0.54	99	1.35 (1.01—1.81)	9.41 ± 0.52	57	1.20 (0.82—1.73)	9.75 ± 0.52	42	1.64 (1.03—2.62)
Q4	11.85 ± 1.33	79	1.18 (0.87—1.62)	11.67 ± 1.34	49	1.12 (0.75—1.66)	12.01 ± 1.31	30	1.31 (0.78—2.20)
***p* for trend**			0.13			0.74			0.13
MUFA									
Q1	6.10 ± 0.96	92	1.00 (ref)	5.87 ± 0.93	57	1.00 (ref)	6.30 ± 0.93	35	1.00 (ref)
Q2	7.79 ± 0.43	82	0.98 (0.73—1.32)	7.59 ± 0.40	46	0.86 (0.58—1.26)	7.94 ± 0.39	36	1.17 (0.74—1.87)
Q3	9.11 ± 0.43	98	1.25 (0.94—1.67)	8.94 ± 0.41	61	1.20 (0.83—1.72)	9.25 ± 0.40	37	1.33 (0.83—2.12)
Q4	10.88 ± 1.09	73	1.03 (0.75—1.41)	10.73 ± 1.10	48	1.02 (0.69—1.51)	10.99 ± 1.07	25	1.00 (0.59—1.70)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
***p* for trend**			0.30			0.39			0.59
PUFA									
Q1	3.24 ± 0.55	109	1.00 (ref)	3.09 ± 0.52	63	1.00 (ref)	3.38 ± 0.54	46	1.00 (ref)
Q2	4.26 ± 0.31	71	0.72 (0.54—0.98)	4.08 ± 0.25	46	0.78 (0.54—1.15)	4.42 ± 0.27	25	0.63 (0.38—1.02)
Q3	5.27 ± 0.43	86	0.93 (0.70—1.24)	5.03 ± 0.34	52	0.93 (0.64—1.36)	5.45 ± 0.39	34	0.92 (0.58—1.44)
Q4	7.30 ± 1.48	79	0.88 (0.65—1.18)	6.90 ± 1.38	51	0.93 (0.64—1.36)	7.62 ± 1.49	28	0.76 (0.47—1.24)
***p* for trend**			0.19			0.65			0.26
Total n-3									
Q1	0.36 ± 0.055	91	1.00 (ref)	0.34 ± 0.051	54	1.00 (ref)	0.38 ± 0.054	37	1.00 (ref)
Q2	0.45 ± 0.030	77	0.90 (0.66—1.22)	0.43 ± 0.022	42	0.80 (0.54—1.20)	0.47 ± 0.023	35	1.04 (0.65—1.65)
Q3	0.53 ± 0.033	93	1.09 (0.82—1.46)	0.51 ± 0.025	67	1.26 (0.88—1.80)	0.55 ± 0.026	26	0.81 (0.49—1.34)
Q4	0.67 ± 0.18	84	1.01 (0.75—1.36)	0.64 ± 0.16	49	0.93 (0.63—1.37)	0.69 ± 0.19	35	1.12 (0.70—1.79)
***p* for trend**			0.66			0.12			0.64
Marine n-3									
Q1	0.092 ± 0.032	96	1.00 (ref)	0.087 ± 0.029	56	1.00 (ref)	0.097 ± 0.033	40	1.00 (ref)
Q2	0.15 ± 0.017	72	0.76 (0.56—1.04)	0.15 ± 0.015	45	0.79 (0.53—1.17)	0.16 ± 0.016	27	0.71 (0.44—1.17)
Q3	0.21 ± 0.019	91	0.95 (0.72—1.27)	0.20 ± 0.017	57	0.99 (0.69—1.44)	0.21 ± 0.018	34	0.89 (0.56—1.40)
Q4	0.29 ± 0.072	86	0.88 (0.66—1.18)	0.28 ± 0.071	54	0.92 (0.63—1.34)	0.30 ± 0.072	32	0.82 (0.51—1.31)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
***p* for trend**			0.34			0.63			0.59
Non-marine n-3									
Q1	0.21 ± 0.033	104	1.00 (ref)	0.21 ± 0.030	64	1.00 (ref)	0.22 ± 0.032	40	1.00 (ref)
Q2	0.28 ± 0.019	79	0.83 (0.62—1.12)	0.26 ± 0.015	52	0.86 (0.59—1.24)	0.29 ± 0.015	27	0.78 (0.48—1.28)
Q3	0.33 ± 0.021	72	0.79 (0.58—1.07)	0.31 ± 0.017	44	0.74 (0.50—1.10)	0.34 ± 0.018	28	0.85 (0.52—1.39)
Q4	0.42 ± 0.18	90	1.00 (0.75—1.34)	0.40 ± 0.16	52	0.87 (0.60—1.26)	0.44 ± 0.19	38	1.20 (0.76—1.90)
***p* for trend**			0.28			0.52			0.32
Total n-6									
Q1	2.82 ± 0.49	108	1.00 (ref)	2.69 ± 0.46	61	1.00 (ref)	2.95 ± 0.48	47	1.00 (ref)
Q2	3.75 ± 0.29	72	0.74 (0.55—1.00)	3.59 ± 0.23	48	0.85 (0.58—1.24)	3.89 ± 0.25	24	0.59 (0.36—0.97)
Q3	4.70 ± 0.41	85	0.93 (0.70—1.24)	4.47 ± 0.32	51	0.95 (0.65—1.39)	4.87 ± 0.38	34	0.89 (0.57—1.40)
Q4	6.64 ± 1.40	80	0.89 (0.66—1.20)	6.26 ± 1.30	52	0.99 (0.67—1.44)	6.95 ± 1.40	28	0.74 (0.46—1.20)
***p* for trend**			0.27			0.83			0.18
Total animal fat									
Q1	4.57 ± 1.27	98	1.00 (ref)	4.82 ± 1.30	57	1.00 (ref)	4.41 ± 1.21	41	1.00 (ref)
Q2	7.14 ± 0.70	82	0.91 (0.68—1.22)	7.49 ± 0.64	52	0.98 (0.67—1.42)	6.87 ± 0.61	30	0.82 (0.51—1.32)
Q3	9.31 ± 0.78	89	1.06 (0.80—1.42)	9.70 ± 0.71	49	0.98 (0.66—1.44)	8.99 ± 0.66	40	1.20 (0.77—1.87)
Q4	12.40 ± 2.23	76	0.99 (0.73—1.35)	12.93 ± 2.23	54	1.18 (0.80—1.73)	11.93 ± 2.12	22	0.71 (0.42—1.22)
***p* for trend**			0.80			0.74			0.20

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Total red meat fat									
Q1	1.08 ± 0.49	102	1.00 (ref)	1.25 ± 0.51	49	1.00 (ref)	0.96 ± 0.44	53	1.00 (ref)
Q2	2.20 ± 0.34	83	0.86 (0.64—1.14)	2.40 ± 0.30	51	1.10 (0.74—1.63)	2.05 ± 0.29	32	0.63 (0.41—0.99)
Q3	3.28 ± 0.43	80	0.86 (0.64—1.15)	3.52 ± 0.38	50	1.11 (0.74—1.64)	3.08 ± 0.36	30	0.63 (0.40—0.98)
Q4	5.08 ± 1.58	80	0.90 (0.67—1.22)	5.40 ± 1.63	62	1.44 (0.98—2.11)	4.81 ± 1.47	18	0.40 (0.23—0.68)
***p* for trend**			0.68			0.26			0.005
Total plant fat									
Q1	11.65 ± 2.02	84	1.00 (ref)	10.74 ± 1.76	57	1.00 (ref)	12.61 ± 1.85	27	1.00 (ref)
Q2	15.06 ± 1.23	88	1.12 (0.83—1.51)	14.03 ± 0.80	52	0.94 (0.64—1.37)	15.92 ± 0.79	36	1.49 (0.90—2.45)
Q3	17.80 ± 1.28	93	1.26 (0.94—1.70)	16.71 ± 0.83	53	1.00 (0.68—1.45)	18.63 ± 0.86	40	1.81 (1.11—2.96)
Q4	21.74 ± 2.92	80	1.16 (0.85—1.59)	20.48 ± 2.72	50	1.01 (0.68—1.48)	22.52 ± 2.76	30	1.46 (0.86—2.48)
***p* for trend**			0.49			0.98			0.13
Total cholesterol									
Q1	62.53 ± 15.42	96	1.00 (ref)	62.88 ± 15.37	53	1.00 (ref)	62.30 ± 15.45	43	1.00 (ref)
Q2	92.04 ± 7.33	79	0.92 (0.68—1.24)	93.16 ± 7.37	43	0.90 (0.60—1.34)	90.98 ± 7.16	36	0.95 (0.61—1.48)
Q3	116.95 ± 8.35	84	1.04 (0.78—1.40)	118.83 ± 8.56	56	1.22 (0.84—1.78)	115.49 ± 7.85	28	0.81 (0.50—1.31)
Q4	156.29 ±	86	1.13	161.28 ±	60	1.36	152.25 ±	26	0.80

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
	42.14		(0.84—1.51)	47.29		(0.93—1.98)	36.47		(0.49—1.32)
***p* for trend**			0.63			0.15			0.75

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 59. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of antioxidant intake and the risk of non-cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
Total selenium (µg/kcal)									
Q1	53.81 ± 5.07	94	1.00 (ref)	53.76 ± 4.85	53	1.00 (ref)	53.83 ± 5.25	47	1.00 (ref)
Q2	61.56 ± 1.88	64	0.67 (0.48—0.91)	61.09 ± 1.75	36	0.67 (0.44—1.02)	61.96 ± 1.90	41	0.66 (0.41—1.07)
Q3	67.82 ± 2.11	94	0.95 (0.72—1.27)	67.11 ± 1.94	62	1.11 (0.77—1.61)	68.37 ± 2.07	28	0.74 (0.47—1.18)
Q4	77.31 ± 8.75	93	0.90 (0.68—1.21)	76.03 ± 7.95	61	1.07 (0.73—1.55)	78.19 ± 9.23	32	0.70 (0.44—1.10)
***p* for trend**			0.07			0.08			0.29
Total carotenoids (µg/kcal)									
Q1	1771.00 ± 499.15	107	1.00 (ref)	1569.97 ± 430.42	64	1.00 (ref)	1980.15 ± 476.48	43	1.00 (ref)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q2	2795.98 ± 376.33	82	0.86 (0.64—1.14)	2524.97 ± 256.31	51	0.86 (0.59—1.25)	3045.52 ± 283.47	31	0.82 (0.52—1.30)
Q3	3844.34 ± 487.95	78	0.89 (0.66—1.19)	3486.02 ± 340.25	53	0.97 (0.68—1.41)	4125.13 ± 385.84	25	0.73 (0.44—1.20)
Q4	5811.57 ± 1948.95	78	0.99 (0.74—1.34)	5265.59 ± 1754.35	44	0.90 (0.61—1.33)	6201.37 ± 1980.48	34	1.09 (0.69—1.74)
***p* for trend**			0.66			0.85			0.37
α-carotene (μg/kcal)									
Q1	29.86 ± 20.52	96	1.00 (ref)	20.07 ± 13.44	62	1.00 (ref)	42.03 ± 21.37	34	1.00 (ref)
Q2	85.81 ± 22.09	84	0.87 (0.65—1.17)	68.89 ± 13.81	49	0.76 (0.52—1.11)	100.43 ± 16.50	35	1.06 (0.66—1.71)
Q3	153.81 ± 36.78	84	0.90 (0.67—1.21)	126.96 ± 23.53	56	0.88 (0.61—1.27)	176.40 ± 30.25	28	0.88 (0.53—1.46)
Q4	332.95 ± 224.87	81	0.93 (0.69—1.25)	278.20 ± 187.03	45	0.75 (0.51—1.11)	375.39 ± 239.39	36	1.23 (0.77—1.98)
***p* for trend**			0.82			0.40			0.61
β-carotene (μg/kcal)									
Q1	635.74 ± 201.97	94	1.00 (ref)	538.81 ± 160.12	55	1.00 (ref)	747.12 ± 186.11	39	1.00 (ref)
Q2	1038.41 ± 171.11	97	1.12 (0.84—1.48	898.74 ± 97.04	65	1.25 (0.87—1.80)	1164.68 ± 116.11	32	0.90 (0.56—1.43)
Q3	1465.99 ± 232.05	69	0.85 (0.62—1.16)	1275.45 ± 132.95	44	0.89 (0.60—1.33)	1626.14 ± 164.74	25	0.75 (0.46—1.25)
Q4	2299.51 ± 880.12	85	1.13 (0.84—1.51)	1975.03 ± 741.98	48	1.04 (0.70—1.53)	2521.20 ± 892.44	37	1.22 (0.77—1.93)
***p* for trend**			0.26			0.35			0.29
β-cryptoxanthin									

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
(µg/kcal)									
Q1	12.06 ± 14.35	103	1.00 (ref)	13.28 ± 14.40	57	1.00 (ref)	10.73 ± 14.30	46	1.00 (ref)
Q2	71.46 ± 17.14	81	0.92 (0.69—1.24)	69.13 ± 15.88	52	1.01 (0.69—1.48)	73.50 ± 17.87	29	0.78 (0.49—1.25)
Q3	142.32 ± 28.18	93	1.12 (0.84—1.50)	136.38 ± 26.63	61	1.23 (0.85—1.78)	147.06 ± 28.41	32	0.93 (0.58—1.47)
Q4	323.55 ± 283.03	68	0.81 (0.59—1.10)	309.38 ± 254.88	42	0.82 (0.55—1.24)	333.47 ± 302.25	26	0.74 (0.45—1.22)
***p* for trend**			0.20			0.25			0.59
Lycopene (µg/kcal)									
Q1	90.53 ± 61.09	123	1.00 (ref)	105.40 ± 65.89	72	1.00 (ref)	80.26 ± 54.58	51	1.00 (ref)
Q2	312.87 ± 79.75	74	0.70 (0.52—0.93)	345.30 ± 78.00	50	0.80 (0.55—1.15)	287.52 ± 72.27	24	0.55 (0.34—0.90)
Q3	627.01 ± 119.05	74	0.77 (0.57—1.03)	657.09 ± 111.43	44	0.76 (0.52—1.11)	603.63 ± 119.41	30	0.76 (0.48—1.21)
Q4	1311.77 ± 1210.93	74	0.89 (0.66—1.20)	1396.34 ± 1196.84	46	0.93 (0.64—1.37)	1245.72 ± 1219.55	28	0.81 (0.50—1.30)
***p* for trend**			0.07			0.43			0.12
Lutein (µg/kcal)									
Q1	597.01 ± 173.40	88	1.00 (ref)	520.16 ± 139.35	53	1.00 (ref)	688.74 ± 163.54	35	1.00 (ref)
Q2	939.52 ± 143.27	90	1.12 (0.83—1.50)	825.32 ± 81.85	51	1.03 (0.70—1.52)	1045.90 ± 97.82	39	1.23 (0.78—1.94)
Q3	1296.07 ± 191.42	86	1.13 (0.84—1.52)	1132.96 ± 110.31	58	1.24 (0.86—1.81)	1427.47 ± 134.42	28	0.93 (0.56—1.53)
Q4	1949.59 ± 639.28	81	1.10 (0.81—1.50)	1684.55 ± 518.06	50	1.10 (0.75—1.63)	2128.98	31	1.07 (0.66—1.75)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
***p* for trend**			0.85			0.67			0.69
Total vitamin C (mg/kcal)									
Q1	21.66 ± 6.76	108	1.00 (ref)	19.99 ± 6.50	62	1.00 (ref)	23.02 ± 6.68	46	1.00 (ref)
Q2	39.63 ± 5.71	80	0.86 (0.64—1.15)	37.35 ± 4.99	49	0.87 (0.59—1.26)	41.60 ± 5.50	31	0.82 (0.52—1.30)
Q3	60.24 ± 8.33	82	0.98 (0.73—1.31)	56.55 ± 6.84	57	1.10 (0.76—1.58)	63.40 ± 8.09	25	0.75 (0.45—1.23)
Q4	102.73 ± 151.60	75	0.94 (0.69—1.27)	94.37 ± 127.18	44	0.87 (0.58—1.30)	108.74 ± 167.54	31	0.99 (0.61—1.59)
***p* for trend**			0.76			0.56			0.60
Total vitamin E (mg α-tocopherol equivalents/kcal)									
Q1	2.59 ± 0.47	94	1.00 (ref)	2.38 ± 0.40	56	1.00 (ref)	2.84 ± 0.43	38	1.00 (ref)
Q2	3.41 ± 0.31	86	1.02 (0.76—1.37)	3.14 ± 0.19	57	1.11 (0.77—1.61)	3.64 ± 0.20	29	0.87 (0.54—1.42)
Q3	4.10 ± 0.34	85	1.10 (0.82—1.47)	3.80 ± 0.21	47	0.96 (0.65—1.43)	4.33 ± 0.23	38	1.28 (0.81—2.02)
Q4	5.25 ± 64.00	80	1.10 (0.81—1.49)	4.85 ± 54.66	52	1.12 (0.76—1.65)	5.49 ± 70.52	28	1.01 (0.62—1.67)
***p* for trend**			0.90			0.83			0.46
Total soy isoflavones (mg/kcal)									
Q1	3.63 ± 1.65	86	1.00 (ref)	3.25 ± 1.45	56	1.00 (ref)	4.00 ± 1.72	30	1.00 (ref)
Q2	7.74 ± 1.35	89	1.08 (0.81—1.46)	7.02 ± 1.07	53	0.97 (0.67—1.41)	8.37 ± 1.24	36	1.28 (0.79—2.09)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q3	12.24 ± 1.87	93	1.18 (0.88—1.59)	11.12 ± 1.46	53	1.00 (0.68—1.46)	13.15 ± 1.66	40	1.52 (0.94—2.45)
Q4	20.72 ± 9.85	77	0.99 (0.73—1.36)	19.12 ± 8.76	50	0.94 (0.64—1.39)	22.00 ± 10.39	27	1.07 (0.63—1.81)
***p* for trend**			0.64			0.99			0.31
Genistein (mg/kcal)									
Q1	1.70 ± 0.76	84	1.00 (ref)	1.52 ± 0.67	55	1.00 (ref)	1.88 ± 0.80	29	1.00 (ref)
Q2	3.58 ± 0.62	96	1.20 (0.90—1.62)	3.24 ± 0.49	58	1.09 (0.75—1.58)	3.87 ± 0.56	38	1.41 (0.87—2.29)
Q3	5.63 ± 0.85	89	1.15 (0.85—1.55)	5.10 ± 0.66	50	0.95 (0.64—1.39)	6.04 ± 0.75	39	1.53 (0.94—2.49)
Q4	9.46 ± 4.37	76	1.01 (0.74—1.38)	8.72 ± 3.90	49	0.94 (0.64—1.39)	10.04 ± 4.59	27	1.11 (0.65—1.88)
***p* for trend**			0.51			0.87			0.27
Daidzein (mg/kcal)									
Q1	1.69 ± 0.78	88	1.00 (ref)	1.52 ± 0.69	56	1.00 (ref)	1.86 ± 0.81	32	1.00 (ref)
Q2	3.65 ± 0.64	88	1.05 (0.78—1.41)	3.31 ± 0.52	52	0.96 (0.65—1.40)	3.96 ± 0.59	36	1.21 (0.75—1.95)
Q3	5.81 ± 0.90	92	1.14 (0.85—1.54)	5.28 ± 0.70	55	1.04 (0.72—1.52)	6.24 ± 0.80	37	1.31 (0.81—2.12)
Q4	9.92 ± 4.84	77	0.97 (0.71—1.32)	9.14 ± 4.29	49	0.92 (0.63—1.36)	10.55 ± 5.12	28	1.04 (0.62—1.73)
***p* for trend**			0.72			0.94			0.65
Glycitein (mg/kcal)									
Q1	0.24 ± 0.11	89	1.00 (ref)	0.21 ± 0.096	57	1.00 (ref)	0.26 ± 0.11	32	1.00 (ref)
Q2	0.51 ±	86	1.01	0.46 ±	52	0.94	0.55 ±	34	1.14

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
	0.087		(0.75—1.36)	0.070		(0.64—1.36)	0.080		(0.70—1.85)
Q3	0.80 ± 0.12	94	1.15 (0.86—1.55)	0.73 ± 0.096	55	1.02 (0.70—1.49)	0.86 ± 0.11	39	1.38 (0.86—2.22)
Q4	1.36 ± 0.65	76	0.94 (0.69—1.29)	1.25 ± 0.58	48	0.89 (0.60—1.31)	1.44 ± 0.69	28	1.04 (0.62—1.73)
***p* for trend**			0.60			0.88			0.52
Total isothiocyanate (μmol/kcal)									
Q1	2.39 ± 0.84	83	1.00 (ref)	2.06 ± 0.71	54	1.00 (ref)	2.71 ± 0.83	29	1.00 (ref)
Q2	4.26 ± 0.68	94	1.22 (0.91—1.65)	3.79 ± 0.48	55	1.08 (0.74—1.58)	4.68 ± 0.54	39	1.46 (0.90—2.37)
Q3	6.25 ± 0.92	88	1.22 (0.90—1.65)	5.59 ± 0.66	56	1.19 (0.82—1.73)	6.77 ± 0.75	32	1.26 (0.76—2.09)
Q4	10.08 ± 4.36	80	1.16 (0.85—1.58)	9.08 ± 3.79	56	1.03 (0.69—1.53)	10.81 ± 4.58	33	1.36 (0.83—2.26)
***p* for trend**			0.52			0.82			0.46

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 60. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for tea and coffee intakes and the risk of non-cardia gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Black tea (cups/month)									
Q1	0.00	225	1.00 (ref)	0.00	130	1.00 (ref)	0.00	95	1.00 (ref)
Q2	2.00 ± 1.14	46	1.10 (0.80—1.51)	2.00 ± 1.14	24	0.87 (0.56—1.34)	2.00 ± 1.13	22	1.50 (0.94—2.40)
Q3	10.70 ± 4.48	27	0.71 (0.47—1.06)	10.70 ± 4.61	19	0.66 (0.41—1.07)	10.70 ± 4.30	8	0.81 (0.39—1.67)
Q4	30.00 ± 26.28	47	1.18 (0.85—1.62)	30.00 ± 27.73	39	1.21 (0.84—1.74)	30.00 ± 23.36	8	0.94 (0.46—1.95)
***p* for trend**			0.19			0.16			0.30
Green tea (cups/month)									
Q1	0.00	211	1.00 (ref)	0.00	125	1.00 (ref)	0.00	86	1.00 (ref)
Q2	2.00 ± 1.11	48	0.80 (0.59—1.10)	2.00 ± 1.11	23	0.60 (0.39—0.94)	2.00 ± 1.10	25	1.13 (0.72—1.77)
Q3	10.70 ± 4.28	36	0.97 (0.68—1.39)	10.70 ± 4.44	23	0.83 (0.53—1.31)	10.70 ± 4.09	13	1.24 (0.69—2.23)
Q4	30.00 ± 40.71	50	1.07 (0.78—1.47)	75.00 ± 42.47	41	1.09 (0.76—1.56)	30.00 ± 38.12	9	0.79 (0.40—1.59)
***p* for trend**			0.50			0.10			0.72
Any tea (cups/month)									
Q1	0.00	149	1.00 (ref)	0.00	81	1.00 (ref)	0.00	68	1.00 (ref)
Q2	4.00 ± 1.83	61	0.89 (0.66—1.19)	4.00 ± 1.87	30	0.70 (0.46—1.06)	4.00 ± 1.79	31	1.16 (0.75—1.77)
Q3	10.70 ± 4.96	48	0.85 (0.61—1.18)	12.70 ± 5.09	30	0.72 (0.47—1.09)	10.70 ± 4.80	18	1.10 (0.65—1.86)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q4	40.70 ± 39.87	87	1.04 (0.79—1.36)	40.70 ± 41.56	71	1.03 (0.74—1.42)	34.30 ± 37.07	16	0.84 (0.48—1.45)
***p* for trend**			0.61			0.13			0.74
Coffee (cups/week)									
Never	0.00	64	1.00 (ref)	0.00	36	1.00 (ref)	0.00	28	1.00 (ref)
Monthly	0.46 ± 0.15	6	0.95 (0.41—2.18)	0.46 ± 0.15	3	0.84 (0.26—2.72)	0.46 ± 0.15	3	1.11 (0.34—3.64)
Weekly	2.50 ± 1.46	27	0.89 (0.57—1.40)	2.50 ± 1.45	11	0.62 (0.32—1.22)	2.50 ± 1.47	16	1.32 (0.72—2.45)
1 cup/day	7.02 ± 0.79	109	0.89 (0.65—1.21)	7.02 ± 0.83	67	1.07 (0.71—1.60)	7.02 ± 0.77	42	0.71 (0.44—1.15)
2-3 cups/day	17.56 ± 1.96	122	1.11 (0.81—1.50)	17.56 ± 1.97	84	1.26 (0.85—1.87)	17.56 ± 1.96	38	0.97 (0.59—1.59)
≥4 cups/day	31.60 ± 5.19	17	1.09 (0.63—1.87)	31.60 ± 4.68	11	1.07 (0.54—2.11)	31.60 ± 5.95	6	1.42 (0.58—3.48)
***p* for trend**			0.68			0.35			0.28

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 61. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fish intake and the risk of unspecified gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
Total fish and shellfish									
Q1	17.60 ± 6.04	25	1.00 (ref)	16.49 ± 5.49	15	1.00 (ref)	18.67 ± 6.34	10	1.00 (ref)
Q2	29.10 ± 3.25	30	1.24 (0.73—2.11)	27.61 ± 2.81	13	0.86 (0.41—1.82)	30.46 ± 3.02	17	1.78 (0.81—3.89)
Q3	39.46 ± 3.80	24	0.97 (0.55—1.70)	37.46 ± 3.26	13	0.85 (0.40—1.78)	41.06 ± 3.43	11	1.12 (0.47—2.64)
Q4	55.58 ± 13.99	22	0.84 (0.47—1.50)	53.05 ± 13.55	14	0.88 (0.42—1.83)	57.42 ± 14.01	8	0.77 (0.30—1.95)
***p* for trend**			0.57			0.97			0.20
Fresh fish and shellfish									
Q1	15.91 ± 5.73	26	1.00 (ref)	14.80 ± 5.18	15	1.00 (ref)	17.08 ± 6.02	11	1.00 (ref)
Q2	27.27 ± 3.24	27	1.07 (0.62—1.83)	25.73 ± 2.76	13	0.86 (0.41—1.81)	28.60 ± 3.00	14	1.32 (0.60—2.92)
Q3	37.54 ± 3.81	26	1.01 (0.59—1.74)	35.45 ± 3.23	13	0.84 (0.40—1.77)	39.19 ± 3.41	13	1.20 (0.54—2.69)
Q4	53.50 ± 14.01	22	0.80 (0.45—1.42)	50.85 ± 13.55	14	0.88 (0.42—1.83)	55.52 ± 14.02	8	0.69 (0.27—1.71)
***p* for trend**			0.78			0.97			0.49
Preserved fish and shellfish									
Q1	0.16 ± 0.13	25	1.00 (ref)	0.19 ± 0.15	14	1.00 (ref)	0.15 ± 0.12	11	1.00 (ref)
Q2	0.80 ± 0.25	27	1.09 (0.63—1.89)	0.88 ± 0.24	13	0.95(0.45—2.03)	0.73 ± 0.23	14	1.29 (0.58—2.85)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
Q3	1.79 ± 0.37	24	0.97 (0.55—1.70)	1.85 ± 0.36	15	1.07 (0.52—2.24)	1.73 ± 0.36	9	0.84 (0.35—2.04)
Q4	3.75 ± 2.57	25	1.03 (0.59—1.80)	3.83 ± 2.59	13	0.94 (0.44—2.02)	3.69 ± 2.56	12	1.16 (0.51—2.65)
***p* for trend**			0.98			0.98			0.77
Fish, fresh and preserved									
Q1	15.03 ± 5.50	25	1.00 (ref)	13.95 ± 4.96	14	1.00 (ref)	16.11 ± 5.78	11	1.00 (ref)
Q2	25.93 ± 3.13	31	1.27 (0.75—2.15)	24.44 ± 2.69	15	1.05 (0.51—2.18)	27.21 ± 2.86	16	1.49 (0.69—3.22)
Q3	35.90 ± 3.74	22	0.87 (0.49—1.54)	33.82 ± 3.13	13	0.87 (0.41—1.86)	37.57 ± 3.37	9	0.81 (0.34—1.96)
Q4	51.73 ± 13.95	23	0.85 (0.48—1.50)	53.06 ± 13.52	13	0.84 (0.39—1.81)	53.69 ± 13.93	10	0.83 (0.35—1.98)
***p* for trend**			0.43			0.93			0.37
Shellfish, fresh and preserved									
Q1	0.69 ± 0.41	31	1.00 (ref)	0.81 ± 0.43	22	1.00 (ref)	0.61 ± 0.38	9	1.00 (ref)
Q2	1.92 ± 0.36	25	0.93 (0.55—1.57)	2.03 ± 0.34	9	0.46 (0.21—1.01)	1.82 ± 0.35	16	2.10 (0.92—4.79)
Q3	3.26 ± 0.50	22	0.87 (0.50—1.51)	3.34 ± 0.47	13	0.71 (0.35—1.43)	3.19 ± 0.51	9	1.27 (0.50—3.23)
Q4	6.06 ± 3.19	23	0.93 (0.54—1.62)	6.02 ± 2.87	11	0.58 (0.28—1.22)	6.11 ± 3.42	12	1.82 (0.75—4.40)
***p* for trend**			0.97			0.21			0.28

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 62. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of fat intakes and the risk of unspecified gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Total Fat									
Q1	18.57 ± 2.81	33	1.00 (ref)	17.71 ± 2.71	16	1.00 (ref)	19.29 ± 2.71	17	1.00 (ref)
Q2	23.33 ± 1.26	24	0.81 (0.48—1.37)	22.62 ± 1.14	13	0.86 (0.41—1.79)	23.88 ± 1.07	11	0.74 (0.34—1.58)
Q3	26.93 ± 1.23	24	0.89 (0.52—1.51)	26.27 ± 1.10	16	1.13 (0.56—2.26)	27.44 ± 1.09	8	0.62 (0.26—1.46)
Q4	31.60 ± 2.74	20	0.85 (0.48—1.52)	31.05 ± 2.76	10	0.77 (0.34—1.74)	32.02 ± 2.65	10	0.94 (0.42—2.14)
***p* for trend**			0.87			0.79			0.67
Saturated Fat									
Q1	5.92 ± 1.01	33	1.00 (ref)	5.76 ± 0.99	14	1.00 (ref)	6.06 ± 1.01	19	1.00 (ref)
Q2	7.93 ± 0.51	28	0.95 (0.57—1.57)	7.77 ± 0.49	15	1.18 (0.57—2.45)	8.16 ± 0.49	13	0.78 (0.38—1.58)
Q3	9.61 ± 0.54	21	0.76 (0.44—1.33)	9.41 ± 0.52	15	1.24 (0.60—2.58)	9.75 ± 0.52	6	0.39 (0.16—0.99)
Q4	11.85 ± 1.33	19	0.75 (0.42—1.35)	11.67 ± 1.34	11	0.96 (0.43—2.15)	12.01 ± 1.31	8	0.59 (0.25—1.40)
***p* for trend**			0.68			0.89			0.23
MUFA									
Q1	6.10 ± 0.96	32	1.00 (ref)	5.87 ± 0.93	19	1.00 (ref)	6.30 ± 0.93	13	1.00 (ref)
Q2	7.79 ± 0.43	24	0.83 (0.49—1.41)	7.59 ± 0.40	13	0.71 (0.35—1.45)	7.94 ± 0.39	11	0.98 (0.44—2.20)
Q3	9.11 ± 0.43	21	0.79 (0.45—1.38)	8.94 ± 0.41	10	0.58 (0.27—1.26)	9.25 ± 0.40	11	1.11 (0.49—2.50)
Q4	10.88 ± 1.09	24	1.02 (0.59—1.76)	10.73 ± 1.10	13	0.82 (0.40—1.70)	10.99 ± 1.07	11	1.34 (0.58—3.08)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
***p* for trend**			0.75			0.55			0.89
PUFA									
Q1	3.24 ± 0.55	26	1.00 (ref)	3.09 ± 0.52	17	1.00 (ref)	3.38 ± 0.54	9	1.00 (ref)
Q2	4.26 ± 0.31	33	1.44 (0.86—2.42)	4.08 ± 0.25	17	1.08 (0.55—2.12)	4.42 ± 0.27	16	2.12 (0.93—4.83)
Q3	5.27 ± 0.43	20	0.95 (0.52—1.71)	5.03 ± 0.34	8	0.53 (0.23—1.23)	5.45 ± 0.39	12	1.75 (0.73—4.21)
Q4	7.30 ± 1.48	22	1.07 (0.60—1.90)	6.90 ± 1.38	13	0.88 (0.42—1.86)	7.62 ± 1.49	9	1.35 (0.53—3.45)
***p* for trend**			0.39			0.39			0.31
Total n-3									
Q1	0.36 ± 0.055	27	1.00 (ref)	0.34 ± 0.051	14	1.00 (ref)	0.38 ± 0.054	13	1.00 (ref)
Q2	0.45 ± 0.030	28	1.11 (0.65—1.88)	0.43 ± 0.022	12	0.87 (0.40—1.89)	0.47 ± 0.023	16	1.35 (0.65—2.82)
Q3	0.53 ± 0.033	24	0.95 (0.54—1.64)	0.51 ± 0.025	13	0.89 (0.42—1.91)	0.55 ± 0.026	11	0.97 (0.43—2.17)
Q4	0.67 ± 0.18	22	0.88 (0.50—1.56)	0.64 ± 0.16	16	1.11 (0.54—2.30)	0.69 ± 0.19	6	0.55 (0.21—1.45)
***p* for trend**			0.87			0.91			0.30
Marine n-3									
Q1	0.092 ± 0.032	27	1.00 (ref)	0.087 ± 0.029	16	1.00 (ref)	0.097 ± 0.033	11	1.00 (ref)
Q2	0.15 ± 0.017	30	1.15 (0.68—1.94)	0.15 ± 0.015	13	0.82 (0.39—1.70)	0.16 ± 0.016	17	1.60 (0.75—3.43)
Q3	0.21 ± 0.019	24	0.90 (0.52—1.56)	0.20 ± 0.017	12	0.73 (0.35—1.55)	0.21 ± 0.018	12	1.11 (0.49—2.52)
Q4	0.29 ± 0.072	20	0.72 (0.40—1.28)	0.28 ± 0.071	14	0.83 (0.40—1.70)	0.30 ± 0.072	6	0.53 (0.19—1.43)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
***p* for trend**			0.42			0.87			0.13
Non-marine n-3									
Q1	0.21 ± 0.033	28	1.00 (ref)	0.21 ± 0.030	16	1.00 (ref)	0.22 ± 0.032	12	1.00 (ref)
Q2	0.28 ± 0.019	23	0.92 (0.53—1.61)	0.26 ± 0.015	8	0.53 (0.22—1.24)	0.29 ± 0.015	15	1.48 (0.69—3.17)
Q3	0.33 ± 0.021	24	0.99 (0.57—1.72)	0.31 ± 0.017	14	0.92 (0.45—1.91)	0.34 ± 0.018	10	1.05 (0.45—2.45)
Q4	0.42 ± 0.18	26	1.09 (0.63—1.88)	0.40 ± 0.16	17	1.08 (0.53—2.17)	0.44 ± 0.19	9	1.00 (0.42—2.41)
***p* for trend**			0.95			0.39			0.70
Total n-6									
Q1	2.82 ± 0.49	26	1.00 (ref)	2.69 ± 0.46	17	1.00 (ref)	2.95 ± 0.48	9	1.00 (ref)
Q2	3.75 ± 0.29	34	1.49 (0.89—2.49)	3.59 ± 0.23	18	1.13 (0.58—2.21)	3.89 ± 0.25	16	2.14 (0.94—4.86)
Q3	4.70 ± 0.41	18	0.85 (0.46—1.57)	4.47 ± 0.32	6	0.40 (0.16—1.02)	4.87 ± 0.38	12	1.75 (0.73—4.19)
Q4	6.64 ± 1.40	23	1.11 (0.63—1.97)	6.26 ± 1.30	14	0.95 (0.46—1.97)	6.95 ± 1.40	9	1.34 (0.53—3.43)
***p* for trend**			0.22			0.16			0.30
Total animal fat									
Q1	4.57 ± 1.27	33	1.00 (ref)	4.82 ± 1.30	18	1.00 (ref)	4.41 ± 1.21	15	1.00 (ref)
Q2	7.14 ± 0.70	22	0.75 (0.43—1.28)	7.49 ± 0.64	9	0.54 (0.24—1.21)	6.87 ± 0.61	13	1.00 (0.47—2.11)
Q3	9.31 ± 0.78	19	0.71 (0.40—1.26)	9.70 ± 0.71	10	0.65 (0.30—1.42)	8.99 ± 0.66	9	0.78 (0.34—1.81)
Q4	12.40 ± 2.23	27	1.13 (0.67—1.91)	12.93 ± 2.23	18	1.30 (0.66—2.55)	11.93 ± 2.12	9	0.90 (0.38—2.13)
***p* for trend**			0.31			0.12			0.94

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI) ***
Total red meat fat									
Q1	1.08 ± 0.49	29	1.00 (ref)	1.25 ± 0.51	14	1.00 (ref)	0.96 ± 0.44	15	1.00 (ref)
Q2	2.20 ± 0.34	25	0.93 (0.55—1.59)	2.40 ± 0.30	12	0.94 (0.44—2.04)	2.05 ± 0.29	13	0.94 (0.44—1.97)
Q3	3.28 ± 0.43	19	0.75 (0.42—1.33)	3.52 ± 0.38	12	0.96 (0.44—2.09)	3.08 ± 0.36	7	0.54 (0.22—1.33)
Q4	5.08 ± 1.58	28	1.18 (0.69—2.01)	5.40 ± 1.63	17	1.45 (0.70—3.00)	4.81 ± 1.47	11	0.93 (0.42—2.07)
***p* for trend**			0.49			0.60			0.57
Total plant fat									
Q1	11.65 ± 2.02	33	1.00 (ref)	10.74 ± 1.76	17	1.00 (ref)	12.61 ± 1.85	16	1.00 (ref)
Q2	15.06 ± 1.23	20	0.64 (0.67—1.12)	14.03 ± 0.80	11	0.65 (0.30—1.39)	15.92 ± 0.79	9	0.62 (0.27—1.41)
Q3	17.80 ± 1.28	26	0.90 (0.54—1.51)	16.71 ± 0.83	13	0.80 (0.39—1.66)	18.63 ± 0.86	13	0.99 (0.47—2.07)
Q4	21.74 ± 2.92	22	0.81 (0.47—1.41)	20.48 ± 2.72	14	0.90 (0.44—1.85)	22.52 ± 2.76	8	0.66 (0.28—1.57)
***p* for trend**			0.47			0.72			0.56
Total cholesterol									
Q1	62.53 ± 15.42	30	1.00 (ref)	62.88 ± 15.37	16	1.00 (ref)	62.30 ± 15.45	14	1.00 (ref)
Q2	92.04 ± 7.33	20	0.77 (0.44—1.35)	93.16 ± 7.37	11	0.78 (0.36—1.69)	90.98 ± 7.16	9	0.75 (0.32—1.74)
Q3	116.95 ± 8.35	23	0.96 (0.55—1.66)	118.83 ± 8.56	13	0.98 (0.47—2.04)	115.49 ± 7.85	10	0.93 (0.41—2.13)
Q4	156.29 ± 42.14	28	1.26 (0.74—2.13)	161.28 ± 47.29	15	1.15 (0.56—2.36)	152.25 ± 36.47	13	1.37 (0.63—2.97)

	Overall			Men			Women		
	Median intake (g/kcal, ± SD)	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *	**Median intake (g/kcal, ± SD)**	**Cases, n**	**HR (95% CI)** *
***p* for trend**			0.41			0.81			0.57

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 63. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for quartiles of antioxidant intake and the risk of unspecified gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Total selenium (μg/kcal)									
Q1	53.81 ± 5.07	12	1.00 (ref)	53.76 ± 4.85	11	1.00 (ref)	53.83 ± 5.25	10	1.00 (ref)
Q2	61.56 ± 1.88	28	1.31 (0.74—2.30)	61.09 ± 1.75	15	1.37 (0.63—2.98)	61.96 ± 1.90	13	1.26 (0.55—2.87)
Q3	67.82 ± 2.11	27	1.21 (0.68—2.15)	67.11 ± 1.94	14	1.21 (0.55—2.66)	68.37 ± 2.07	13	1.21 (0.53—2.77)
Q4	77.31 ± 8.75	25	1.03 (0.58—1.86)	76.03 ± 7.95	15	1.20 (0.55—2.65)	78.19 ± 9.23	10	0.84 (0.35—2.04)
***p* for trend**			0.75			0.89			0.77
Total carotenoids (μg/kcal)									
Q1	1771.00 ± 499.15	38	1.00 (ref)	1569.97 ± 430.42	17	1.00 (ref)	1980.15 ± 476.48	21	1.00 (ref)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
Q2	2795.98 ± 376.33	20	0.60 (0.35—1.04)	2524.97 ± 256.31	13	0.87 (0.42—1.81)	3045.52 ± 283.47	7	0.38 (0.16—0.89)
Q3	3844.34 ± 487.95	23	0.76 (0.45—1.29)	3486.02 ± 340.25	13	0.95 (0.46—1.96)	4125.13 ± 385.84	10	0.59 (0.28—1.27)
Q4	5811.57 ± 1948.95	20	0.75 (0.43—1.31)	5265.59 ± 1754.35	12	1.00 (0.47—2.12)	6201.37 ± 1980.48	9	0.54 (0.23—1.23)
***p* for trend**			0.32			0.98			0.11
α-carotene (μg/kcal)									
Q1	29.86 ± 20.52	30	1.00 (ref)	20.07 ± 13.44	18	1.00 (ref)	42.03 ± 21.37	12	1.00 (ref)
Q2	85.81 ± 22.09	28	0.95 (0.56—1.59)	68.89 ± 13.81	13	0.73 (0.36—1.50)	100.43 ± 16.50	15	1.25 (0.59—2.68)
Q3	153.81 ± 36.78	20	0.69 (0.39—1.22)	126.96 ± 23.53	9	0.50 (0.23—1.12)	176.40 ± 30.25	11	0.96 (0.42—2.18)
Q4	332.95 ± 224.87	23	0.86 (0.50—1.48)	278.20 ± 187.03	15	0.88 (0.44—1.75)	375.39 ± 239.39	8	0.78 (0.32—1.92)
***p* for trend**			0.61			0.39			0.74
β-carotene (μg/kcal)									
Q1	635.74 ± 201.97	33	1.00 (ref)	538.81 ± 160.12	17	1.00 (ref)	747.12 ± 186.11	16	1.00 (ref)
Q2	1038.41 ± 171.11	29	0.96 (0.58—1.59)	898.74 ± 97.04	14	0.90 (0.44—1.83)	1164.68 ± 116.11	15	1.01 (0.50—2.04)
Q3	1465.99 ± 232.05	18	0.64 (0.36—1.15)	1275.45 ± 132.95	10	0.68 (0.31—1.48)	1626.14 ± 164.74	8	0.59 (0.25—1.38)
Q4	2299.51 ± 880.12	21	0.82 (0.47—1.42)	1975.03 ± 741.98	14	1.01 (0.49—2.07)	2521.20 ± 892.44	7	0.57 (0.23—1.40)
***p* for trend**			0.45			0.76			0.39
β-cryptoxanthin									

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
(µg/kcal)									
Q1	12.06 ± 14.35	35	1.00 (ref)	13.28 ± 14.40	19	1.00 (ref)	10.73 ± 14.30	16	1.00 (ref)
Q2	71.46 ± 17.14	19	0.67 (0.38—1.17)	69.13 ± 15.88	10	0.61 (0.28—1.33)	73.50 ± 17.87	9	0.70 (0.31—1.60)
Q3	142.32 ± 28.18	26	0.98 (0.58—1.65)	136.38 ± 26.63	19	1.21 (0.63—2.32)	147.06 ± 28.41	7	0.60 (0.24—1.47)
Q4	323.55 ± 283.03	21	0.78 (0.44—1.36)	309.38 ± 254.88	7	0.43 (0.18—1.03)	333.47 ± 302.25	14	1.19 (0.57—2.51)
***p* for trend**			0.45			0.07			0.39
Lycopene (µg/kcal)									
Q1	90.53 ± 61.09	29	1.00 (ref)	105.40 ± 65.89	16	1.00 (ref)	80.26 ± 54.58	13	1.00 (ref)
Q2	312.87 ± 79.75	29	1.23 (0.73—2.06)	345.30 ± 78.00	14	1.07 (0.52—2.21)	287.52 ± 72.27	15	1.39 (0.66—2.95)
Q3	627.01 ± 119.05	27	1.28 (0.75—2.18)	657.09 ± 111.43	17	1.45 (0.72—2.90)	603.63 ± 119.41	10	1.05 (0.45—2.43)
Q4	1311.77 ± 1210.93	16	0.89 (0.48—1.66)	1396.34 ± 1196.84	8	0.81 (0.34—1.92)	1245.72 ± 1219.55	8	0.97 (0.39—2.39)
***p* for trend**			0.59			0.53			0.78
Lutein (µg/kcal)									
Q1	597.01 ± 173.40	29	1.00 (ref)	520.16 ± 139.35	15	1.00 (ref)	688.74 ± 163.54	14	1.00 (ref)
Q2	939.52 ± 143.27	36	1.37 (0.84—2.24)	825.32 ± 81.85	20	1.47 (0.75—2.88)	1045.90 ± 97.82	16	1.25 (0.61—2.57)
Q3	1296.07 ± 191.42	15	0.61 (0.33—1.15)	1132.96 ± 110.31	7	0.56 (0.23—1.38)	1427.47 ± 134.42	8	0.66 (0.28—1.58)
Q4	1949.59 ± 639.28	21	0.89 (0.51—1.57)	1684.55 ± 518.06	13	1.06 (0.50—2.24)	2128.98	8	0.70 (0.29—1.69)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
***p* for trend**			0.06			0.17			0.39
Total vitamin C (mg/kcal)									
Q1	21.66 ± 6.76	36	1.00 (ref)	19.99 ± 6.50	17	1.00 (ref)	23.02 ± 6.68	19	1.00 (ref)
Q2	39.63 ± 5.71	20	0.67 (0.39—1.16)	37.35 ± 4.99	11	0.74 (0.35—1.60)	41.60 ± 5.50	9	0.57 (0.26—1.28)
Q3	60.24 ± 8.33	28	1.07 (0.64—1.77)	56.55 ± 6.84	18	1.36 (0.69—2.67)	63.40 ± 8.09	10	0.72 (0.33—1.59)
Q4	102.73 ± 151.60	17	0.69 (0.38—1.24)	94.37 ± 127.18	9	0.68 (0.30—1.57)	108.74 ± 167.54	8	0.65 (0.28—1.53)
***p* for trend**			0.25			0.27			0.53
Total vitamin E (mg α-tocopherol equivalents/kcal)									
Q1	2.59 ± 0.47	32	1.00 (ref)	2.38 ± 0.40	19	1.00 (ref)	2.84 ± 0.43	13	1.00 (ref)
Q2	3.41 ± 0.31	28	0.98 (0.59—163)	3.14 ± 0.19	10	0.58 (0.27—1.24)	3.64 ± 0.20	18	1.58 (0.77—3.24)
Q3	4.10 ± 0.34	25	0.96 (0.57—1.63)	3.80 ± 0.21	14	0.85 (0.42—1.71)	4.33 ± 0.23	11	1.08 (0.48—2.43)
Q4	5.25 ± 64.00	16	0.66 (0.36—1.22)	4.85 ± 54.66	12	0.75 (0.36—1.58)	5.49 ± 70.52	4	0.44 (0.14—1.38)
***p* for trend**			0.56			0.56			0.13
Total soy isoflavones (mg/kcal)									
Q1	3.63 ± 1.65	35	1.00 (ref)	3.25 ± 1.45	21	1.00 (ref)	4.00 ± 1.72	14	1.00 (ref)
Q2	7.74 ± 1.35	19	0.57 (0.33—1.00)	7.02 ± 1.07	6	0.30 (0.12—0.73)	8.37 ± 1.24	13	0.98 (0.46—2.08)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Q3	12.24 ± 1.87	26	0.82 (0.49—1.37)	11.12 ± 1.46	16	0.81 (0.42—1.57)	13.15 ± 1.66	10	0.81 (0.36—1.83)
Q4	20.72 ± 9.85	21	0.67 (0.39—1.16)	19.12 ± 8.76	12	0.60 (0.29—1.24)	22.00 ± 10.39	9	0.76 (0.32—1.77)
***p* for trend**			0.21			0.06			0.89
Genistein (mg/kcal)									
Q1	1.70 ± 0.76	36	1.00 (ref)	1.52 ± 0.67	21	1.00 (ref)	1.88 ± 0.80	15	1.00 (ref)
Q2	3.58 ± 0.62	18	0.53 (0.30—0.93)	3.24 ± 0.49	6	0.30 (0.12—0.74)	3.87 ± 0.56	12	0.84 (0.39—1.80)
Q3	5.63 ± 0.85	25	0.76 (0.46—1.28)	5.10 ± 0.66	15	0.76 (0.39—1.48)	6.04 ± 0.75	10	0.75 (0.34—1.68)
Q4	9.46 ± 4.37	22	0.69 (0.40—1.18)	8.72 ± 3.90	13	0.66 (0.33—1.33)	10.04 ± 4.59	9	0.71 (0.31—1.63)
***p* for trend**			0.15			0.07			0.84
Daidzein (mg/kcal)									
Q1	1.69 ± 0.78	35	1.00 (ref)	1.52 ± 0.69	21	1.00 (ref)	1.86 ± 0.81	14	1.00 (ref)
Q2	3.65 ± 0.64	19	0.57 (0.33—1.00)	3.31 ± 0.52	6	0.30 (0.12—0.74)	3.96 ± 0.59	13	0.98 (0.46—2.09)
Q3	5.81 ± 0.90	26	0.82 (0.49—1.37)	5.28 ± 0.70	16	0.82 (0.43—1.59)	6.24 ± 0.80	10	0.81 (0.36—1.83)
Q4	9.92 ± 4.84	21	0.67 (0.39—1.17)	9.14 ± 4.29	12	0.61 (0.29—1.25)	10.55 ± 5.12	9	0.76 (0.33—1.77)
***p* for trend**			0.22			0.06			0.89
Glycitein (mg/kcal)									
Q1	0.24 ± 0.11	36	1.00 (ref)	0.21 ± 0.096	21	1.00 (ref)	0.26 ± 0.11	15	1.00 (ref)
Q2	0.51 ±	20	0.58	0.46 ±	7	0.35	0.55 ±	13	0.91

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
	0.087		(0.34—1.01)	0.070		(0.15—0.81)	0.080		(0.43—1.92)
Q3	0.80 ± 0.12	25	0.77 (0.46—1.28)	0.73 ± 0.096	15	0.77 (0.39—1.50)	0.86 ± 0.11	10	0.75 (0.33—1.68)
Q4	1.36 ± 0.65	20	0.62 (0.36—1.08)	1.25 ± 0.58	12	0.60 (0.29—1.24)	1.44 ± 0.69	8	0.62 (0.26—1.48)
***p* for trend**			0.18			0.09			0.72
Total isothiocyanate (μmol/kcal)									
Q1	2.39 ± 0.84	36	1.00 (ref)	2.06 ± 0.71	23	1.00 (ref)	2.71 ± 0.83	13	1.00 (ref)
Q2	4.26 ± 0.68	26	0.78 (0.47—1.30)	3.79 ± 0.48	6	0.29 (0.12—0.70)	4.68 ± 0.54	20	1.66 (0.82—3.34)
Q3	6.25 ± 0.92	21	0.68 (0.39—1.16)	5.59 ± 0.66	12	0.62 (0.31—1.25)	6.77 ± 0.75	9	0.77 (0.33—1.82)
Q4	10.08 ± 4.36	18	0.61 (0.34—1.08)	9.08 ± 3.79	14	0.74 (0.38—1.46)	10.81 ± 4.58	4	0.37 (0.12—1.13)
***p* for trend**			0.30			0.05			0.02

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)

Table 64. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for tea and coffee intakes and the risk of unspecified gastric cancer in the Singapore Chinese Health Study.

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *	**Median intake ± SD**	**Cases, n**	**HR (95% CI)** *
Black tea (cups/month)									
Q1	0.00	63	1.00 (ref)	0.00	31	1.00 (ref)	0.00	32	1.00 (ref)
Q2	2.00 ± 1.14	10	0.90 (0.46—1.76)	2.00 ± 1.14	6	0.95 (0.39—2.28)	2.00 ± 1.13	4	0.83 (0.29—2.37)
Q3	10.70 ± 4.48	13	1.35 (0.74—2.48)	10.70 ± 4.61	8	1.22 (0.56—2.67)	10.70 ± 4.30	5	1.57 (0.61—4.07)
Q4	30.00 ± 26.28	15	1.53 (0.86—2.72)	30.00 ± 27.73	10	1.38 (0.67—2.83)	30.00 ± 23.36	5	1.86 (0.72—4.83)
***p* for trend**			0.40			0.81			0.47
Green tea (cups/month)									
Q1	0.00	56	1.00 (ref)	0.00	29	1.00 (ref)	0.00	27	1.00 (ref)
Q2	2.00 ± 1.11	23	1.53 (0.94—2.50)	2.00 ± 1.11	11	1.31 (0.65—2.62)	2.00 ± 1.10	12	1.79 (0.91—3.55)
Q3	10.70 ± 4.28	10	1.12 (0.57—2.21)	10.70 ± 4.44	8	1.31 (0.60—2.89)	10.70 ± 4.09	2	0.65 (0.15—2.75)
Q4	30.00 ± 40.71	12	1.12 (0.59—2.12)	75.00 ± 42.47	7	0.87 (0.38—2.01)	30.00 ± 38.12	5	1.56 (0.59—4.11)
***p* for trend**			0.40			0.76			0.27
Any tea (cups/month)									
Q1	0.00	36	1.00 (ref)	0.00	17	1.00 (ref)	0.00	19	1.00 (ref)
Q2	4.00 ± 1.83	23	1.48 (0.88—2.51)	4.00 ± 1.87	9	1.04 (0.46—2.35)	4.00 ± 1.79	14	1.93 (0.96—3.86)
Q3	10.70 ± 4.96	16	1.32 (0.73—2.41)	12.70 ± 5.09	12	1.44 (0.69—3.04)	10.70 ± 4.80	4	0.93 (0.32—2.76)

	Overall			Men			Women		
	Median intake ± SD	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***	**Median intake ± SD**	**Cases, n**	**HR (95% CI) ***
Q4	40.70 ± 39.87	26	1.53 (0.91—2.58)	40.70 ± 41.56	17	1.28 (0.65—2.53)	34.30 ± 37.07	9	1.85 (0.83—4.15)
***p* for trend**			0.34			0.76			0.18
Coffee (cups/week)									
Never	0.00	22	1.00 (ref)	0.00	12	1.00 (ref)	0.00	10	1.00 (ref)
Monthly	0.46 ± 0.15	3	1.39 (0.42—4.64)	0.46 ± 0.15	2	1.73 (0.39—7.76)	0.46 ± 0.15	1	1.06 (0.14—8.30)
Weekly	2.50 ± 1.46	12	1.19 (0.59—2.41)	2.50 ± 1.45	6	1.07 (0.40—2.87)	2.50 ± 1.47	6	1.40 (0.51—3.86)
1 cup/day	7.02 ± 0.79	29	0.69 (0.39—1.20)	7.02 ± 0.83	18	0.88 (0.42—1.82)	7.02 ± 0.77	11	0.52 (0.22—1.23)
2-3 cups/day	17.56 ± 1.96	31	0.85 (0.49—1.48)	17.56 ± 1.97	15	0.68 (0.32—1.47)	17.56 ± 1.96	16	1.17 (0.53—2.60)
≥4 cups/day	31.60 ± 5.19	4	0.77 (0.26—2.27)	31.60 ± 4.68	2	0.55 (0.12—2.48)	31.60 ± 5.95	2	1.47 (0.32—6.84)
***p* for trend**			0.56			0.75			0.31

* adjusted for age (years), gender (male/female, in overall model only), interview year (1993-1995/1996-1998), dialect (Hokkien/Cantonese), education (less than secondary/secondary or greater), tobacco smoking (never/ever), alcohol consumption (never/ever), body mass index (<20, 20-<24, 24-<28, or ≥28 kg/m^2), and total energy intake (kcal/day)