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Red and processed meat intake and cancer risk: Results from the prospective NutriNet-Santé cohort study

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Cancer Epidemiology

The International Agency for Research on Cancer (WHO-IARC) classified red meat and processed meat as probably carcinogenic and carcinogenic for humans, respectively. These conclusions were mainly based on studies concerning colorectal cancer, but scientific evidence is still limited for other cancer locations. In this study, we investigated the prospective associations between red and processed meat intakes and overall, breast, and prostate cancer risk. This prospective study included 61,476 men and women of the French NutriNet-Santé cohort (2009–2015) aged ≥35 y and who completed at least three 24 hrs dietary records during the first year of follow-up. The risk of developing cancer was compared across sex-specific quintiles of red and processed meat intakes by multivariable Cox models. 1,609 first primary incident cancer cases were diagnosed during follow-up, among which 544 breast cancers and 222 prostate cancers. Red meat intake was associated with increased risk of overall cancers [$HR_{Q5vs.Q1}$=1.31 (1.10–1.55), p_{trend} = 0.01) and breast cancer ($HR_{Q5vs.Q1}$ = 1.83 (1.33–2.51), p_{trend} = 0.002]. The latter association was observed in both premenopausal [$HR_{Q5vs.Q1}$=2.04 (1.03–4.06)] and postmenopausal women [$HR_{Q5vs.Q1}$=1.79 (1.26-2.55)]. No association was observed between red meat intake and prostate cancer risk. Processed meat intake was relatively low in this study (cut-offs for the 5th quintile = 46 g/d in men and 29 g/d in women) and was not associated with overall, breast or prostate cancer risk. This large cohort study suggested that red meat may be involved carcinogenesis at several cancer locations (other than colon-rectum), in particular breast cancer. These results are consistent with mechanistic evidence from experimental studies.

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The International Agency for Research on Cancer (WHO-IARC) recently classified consumption of processed meat as "carcinogenic to humans" and consumption of red meat as "probably carcinogenic to humans."[1] The World Cancer Research Fund and the American Institute for Cancer Research (WCRF/AICR) recommends consuming <500 g/week of red meat and <50 g/d of processed meat.[2] These conclusions were mainly based on findings concerning colorectal cancer, for which the weight of evidence is considered as convincing.[3,4] Indeed, experimental studies showed that several components of red and/or processed meat act locally on the colorectal mucosa to promote carcinogenesis. Potential carcinogens include heme iron, nitrates and nitrites and mutagenic compounds such as neoformed products generated in red meats and processed meat (heterocyclic amines, polycyclic aromatic hydrocarbons, N-nitroso compounds.[3,5,6] However, these pro-carcinogens may also be involved in more systemic mechanisms,[7–10] suggesting that red and processed meat may impact cancer risk for cancer locations other than colon-rectum.

Despite these mechanistic hypotheses, epidemiological evidence regarding red/processed meat and cancer risk is limited for other cancer locations, and notably for breast and prostate cancers, which are the two main cancer sites in many Western countries.[11,12] In a previous study performed in the

What's new?

Red meat contains multiple substances that are potentially carcinogenic, including nitrates, nitrites, and heterocyclic amines. Its consumption, presumably owing to the presence of these substances, is associated with carcinogenic processes primarily in the colorectal mucosa. The present study shows, however, that red meat intake is also associated with increased risk of cancer overall, as well as with increased breast cancer risk specifically. Breast cancer risk was elevated for both premenopausal and postmenopausal women. The findings indicate that red meat intake affects more than the colorectal mucosa and that its restriction could be important in preventing tumors at other sites.

SU.VI.MAX cohort,[13] we observed that processed meat intake was associated with increased breast cancer risk. This result is consistent with two recent meta-analyses suggesting positive associations with breast cancer risk.[14,15] Since the publication of these meta-analyses, two prospective cohort studies were published. Inoue-Cho *et al.*[16] observed an increased risk of breast cancer in post-menopausal women with high consumption of red or processed meat; and Bertrand *et al.*[17] showed increased breast density in pre-menopausal women associated with high consumption of red meat. In 2014, the World Cancer Research Fund and the American Institute for Cancer Research (WCRF/AICR) observed null results for their meta-analyses of the associations between red and processed meat and prostate cancer risk,[18] consistent with a meta-analysis published in 2015.[19] In contrast, in a pooled analysis of 15 cohort studies published in 2016, Wu *et al.*[20] observed a positive association between red and processed meat and risk of advanced prostate cancer. Thus, the weight of evidence is still considered as "limited" regarding red and processed meat and cancer risk for non-colorectal locations.[3,18,21,22] No consensus has been reached so far and additional prospective studies are needed to more thoroughly elucidate the relationship between red and processed meat intakes and breast or prostate cancer risk.

The objective of this prospective study was to investigate the associations between red meat and processed meat intakes and overall, breast and prostate cancer risk, in a large cohort of French adults with accurate and up-to-date dietary intake data.

Methods

Study population

The NutriNet-Santé study is an ongoing web-based cohort launched in 2009 in France with the objective to study the associations between nutrition and health as well as the determinants of dietary behaviors and nutritional status. This cohort has been previously described in details.[23] Participants aged over 18 years with access to the Internet are continuously recruited since May 2009 among the general population by means of vast multimedia campaigns. All questionnaires are completed online using a dedicated website (www.etude-nutrinet-sante.fr). The NutriNet-Santé study is conducted according to the Declaration of Helsinki guidelines and was approved by the Institutional Review Board of the French Institute for Health and Medical Research (IRB Inserm n°0000388FWA00005831) and the "Commission Nationale de l'Informatique et des Libertés" (CNIL n°908450/n°909216). Electronic informed consent is obtained from each participant (EudraCT no. 2013–000929-31).

Data collection

Dietary data. Dietary intakes were assessed every 6 months through a series of three non-consecutive validated web-based 24 hrs-dietary records, randomly assigned over a 2-week period (2 weekdays and 1 weekend day).[24–26] Participants used a dedicated interface of the study website to declare all foods and beverages consumed during a 24 hrs-period: three main meals (breakfast, lunch, dinner) or any other eating occasion. Portion sizes were estimated using validated photographs.[27] Mean daily energy, alcohol and nutrient intakes were estimated using a published French food composition table (>3,300 items).[28] Amounts consumed from composite dishes were estimated using French recipes validated by food and nutrition professionals. Dietary underreporting was identified on the basis of the method proposed by Black.[29] Red meat intake was defined as fresh, minced and frozen beef, veal, pork, and lamb. Processed meat intake was defined as mostly pork and beef preserved by methods other than freezing, such as salting, smoking, marinating, air-drying or heating and included ham, bacon, sausages, blood sausages, liver pâté, salami, mortadella, tinned meat and others.

Covariates. At inclusion, participants fulfilled a set of five questionnaires related to socio-demographic and lifestyle characteristics[30] (*e.g.*, sex, date of birth, educational level, smoking status, number of children), anthropometrics[31,32] (*e.g.*, height and weight), dietary intakes (see above), physical activity (validated IPAQ questionnaire)[33] and health status (*e.g.*, personal and family history of diseases, medication use including hormonal treatment for menopause and oral contraception, menopausal status).

Case ascertainment

Participants self-declared health events through the yearly health status questionnaire, through a specific check-up questionnaire for health events (every 3 months) or at any time through a specific interface on the study website. Following this declaration, participants were invited to send their

medical records (diagnosis, hospitalization, *etc.*) and, if necessary, the study physicians contacted the participants' treating physician or the medical structures to collect additional information. Then, data were reviewed by an independent physician expert committee for the validation of major health events. Cancer cases were classified using the International Chronic Diseases Classification, 10th Revision, Clinical Modification (ICD-10).[34] In this study, all first primary cancers diagnosed between the inclusion and August 2015 were considered as cases (except basal cell skin carcinoma, which was not considered as cancer).

Statistical analyses

So far, 96,716 subjects without cancer at baseline provided at least three valid 24 hrs-dietary records during their first year of follow-up. Participants aged <35 y ($n = 32{,}882$) were excluded because of a very low susceptibility to develop cancer and so were subjects with a null follow-up ($n = 2{,}358$). Thus, 61,476 subjects were included in the analyses.

Estimated red and processed meat and other dietary intakes were based on the average intake for each subject across all 24 hrs-dietary records available in their first year of follow-up. For all covariates except physical activity, $\leq 5\%$ of values were missing and were imputed to the modal value. For physical activity (13% of missing values), a "missing class" was introduced into the models.

Baseline characteristics of participants were compared across sex-specific quintiles of red and processed meat intake using χ^2 tests or Fisher tests wherever appropriate. We estimated hazard ratios (HR) and 95% confidence intervals (CI) using Cox proportional hazards models, with age as the primary time variable, to characterize the association between sex-specific quintiles of red meat, processed meat and total red and processed meat intake and incidence of overall, breast or prostate cancer risk (the two main cancer locations in the cohort). We confirmed that the assumptions of proportionality were satisfied through examination of the log-log (survival) versus log-time plots. Tests for linear trend were performed using the ordinal score on sex-specific quintiles of intake. Participants contributed person-time until the date of cancer diagnosis, the date of last completed questionnaire, the date of death, or August 31, 2015, whichever occurred first. For cancer site specific analysis, women who reported a cancer other than breast cancer and men who reported a cancer other than prostate cancer during the study period were censored at the date of diagnosis. Analyses were performed according to menopausal status for breast cancer analyses. For these analyses, women contributed person-time in the Cox model until their date of menopause for premenopausal breast cancer analysis or from their date of menopause for postmenopausal breast cancer analysis. Additionally, models restricted to invasive breast cancer cases (excluding *in situ* cases) were tested.

Models were adjusted for age (time-scale), sex (for overall cancers only), BMI (kg/m^2, continuous), height (cm, continuous), physical activity (high, moderate, low, computed following IPAQ recommendations[35]), smoking status (never smokers, former smokers, current smokers), number of 24 hrs-dietary records (continuous), fruits and vegetables intake (g/d, continuous), total lipids intake (g/d, continuous), alcohol intake (g/d, continuous), energy intake (without alcohol, g/d, continuous), family history of cancer (yes/no) and educational level (<high-school degree, <2 years after high-school degree, ≥2 years after high-school degree). Red and processed meat models were mutually adjusted for processed meat and red meat intakes, respectively. For breast cancer analyses, additional adjustments were performed for the number of biological children (continuous), menopausal status at baseline (yes/no), hormonal treatment for menopause at baseline (only for postmenopausal analyses, yes/no) and oral contraception use at baseline (only for premenopausal analyses, yes/no). Since antioxidants may partly counteract lipid peroxidation by heme iron from red and processed meat (*i.e.*, one of the hypothesized mechanisms involved in their potentially procarcingenic effect)[1], we have tested for a potential interaction between fruit and vegetable intake (as a proxy for antioxidant exposure, according to sex-specific median intake) and red and processed meat intake by introducing the product of the two variables into Cox models for each cancer location. Stratified analyses were performed when appropriate (*i.e.*, p-interaction < 0.1).

All tests were two-sided, and $p < 0.05$ was considered statistically significant. SAS version 9.4 (SAS Institute) was used for the analyses.

Results

Between May 2009 and August 2015 (median follow-up time: 4.1 year; 229,835 person-years), 1,609 incident cancer cases were diagnosed, among which 544 breast cancers (169 premenopausal and 375 postmenopausal; 71.6% ER+/PR+, 13.5% ER–/PR–, 14.6% ER+/PR–, 0.3% ER-/PR+; 80.4% invasive and 19.6% *in situ*), 222 prostate cancers (88,46% Gleason score < 7, 11,54% Gleason score ≥7) and 843 other cancers (169 skin (other than basal cell carcinoma), 120 colorectal, 64 lymphomas, 63 lung, 39 thyroid, 38 cervix, 38 bladder, 37 uterus, 35 leukemia, 30 kidney and 210 others).

Mean age at diagnosis was 51.68 y ± 10.14 and mean baseline-to-diagnosis time was 2.43 y ± 1.60. Mean number of 24 hrs dietary records per subject over their first year of follow-up was 4.53 ± 1.61.

Characteristics of the participants according to quintiles of total red and processed meat intakes are described in Table 1. Mean daily red meat intake was 42.9 ± 39.0 g/d (0.4 ± 1.9 g/d in the first quintile, 102.3 ± 33.7 g/d in the fifth quintile). Mean daily processed meat intake was 19.1 ± 23.8 g/d (0 g/d in the first quintile, 56.0 ± 25.9 g/d in the fifth quintile; data not tabulated). Subjects with higher total red and processed meat intake were more likely to be younger, to have a higher body mass index, to smoke, to have higher energy, lipid and alcohol intakes and lower fruit

Table 1. Baseline characteristics of study participants ($n = 61,476$) according to sex-specific quintiles of red and processed meat intake, NutriNet-Santé cohort, France, 2009–2016[1]

	Quintile 1 ($n = 12,292$)	Quintile 2 ($n = 12,298$)	Quintile 3 ($n = 12,303$)	Quintile 4 ($n = 12,287$)	Quintile 5 ($n = 12,296$)
Age, y	51.7 +/− 10.2	52 +/− 10.3	52.2 +/− 10.2	51.9 +/− 10.1	50.6 +/− 9.8
Sex					
Men	3,107 (25.28)	3,111 (25.29)	3,110 (25.28)	3,108 (25.30)	3,110 (25.29)
Women	9,185(74.72)	9,187(74.71)	9,193(74.72)	9,179(74.70)	9,186(74.71)
Height, cm	166.5 +/− 8.2	166.4 +/− 8.1	166.4 +/− 8.2	166.6 +/− 8.2	167.1 +/− 8.3
Body mass index, kg/m^2	23.4 +/− 4.2	23.9 +/− 4.2	24.3 +/− 4.4	24.9 +/− 4.6	25.7 +/− 5.2
Family history of cancer[2], yes	5,429 (44.2)	5,557 (45.2)	5,627 (45.7)	5,570 (45.3)	5,465 (44.4)
Number of children, *n*	1.7 +/− 1.2	1.8 +/− 1.2	1.9 +/− 1.1	1.9 +/− 1.2	1.9 +/− 1.1
Higher education					
No	2,718 (22.1)	2,806 (22.8)	3,034 (24.7)	3,316 (27.0)	3,543 (28.8)
Yes, < 2 years	1,885 (15.3)	1,876 (15.3)	1,785 (14.5)	1,955 (15.9)	2,042 (16.6)
Yes, ≥ 2 years	7,689 (62.6)	7,616 (61.9)	7,484 (60.8)	7,016 (57.1)	6,711 (54.6)
Smoking status					
Current	1,476 (12.0)	1,476 (12.0)	1,542 (12.5)	1,777 (14.5)	2,116 (17.2)
Former	5,063 (41.2)	5,046 (41.0)	5,000 (40.6)	5,042 (41.0)	5,109 (41.6)
Never	5,753 (46.8)	5,776 (47.0)	5,761 (46.8)	5,468 (44.5)	5,071 (41.2)
IPAQ Physical activity level[3]					
High	4,292 (34.9)	4,056 (33)	3,881 (31.5)	3,802 (30.9)	3,628 (29.5)
Moderate	4,524 (36.8)	4,460 (36.3)	4,413 (35.9)	4,290 (34.9)	4,001 (32.5)
Low	2,049 (16.7)	2,316 (18.8)	2,452 (19.9)	2,600 (21.2)	2,870 (23.3)
Processed meat intake, g/d	3.8 +/− 6.1	11.6 +/− 12.2	17.3 +/− 16.7	24.2 +/− 21.6	38.6 +/− 34.6
Red meat intake, g/d	3.6 +/− 6.7	22.3 +/− 13.8	38.5 +/− 17.9	56.2 +/− 23.0	93.9 +/− 42.0
Fruits and vegetables intake, g/d	496.9 +/− 257.7	458.7 +/− 211.6	442.4 +/− 203.0	431.6 +/− 198.8	411.4 +/− 203.5
Energy intake, kcal/d	1,720 +/− 443.9	1,769.1 +/− 425.2	1,805 +/− 433.3	1,844.5 +/− 436.0	1,962.3 +/− 492.1
Total lipid intake, g/d	73 +/− 24.5	76.4 +/− 23.1	79.3 +/− 23.4	82.6 +/− 24.2	90.9 +/− 27.8
Alcohol intake, g/d	6.3 +/− 10.1	7.8 +/− 11.0	8.7 +/− 11.7	9.9 +/− 13.3	11.9 +/− 15.9
Oral contraception, yes	1,021 (11.1)	1,118 (12.2)	1,112 (12.1)	1,176 (12.8)	1,299 (14.1)
Hormonal treatment for menopause, yes	750 (8.2)	830 (9.0)	882 (9.6)	827 (9.0)	699 (7.6)
Menopausal, yes	4,557 (49.6)	4,546 (49.5)	4,698 (51.1)	4,537 (49.4)	4,056 (44.2)

[1]Values are means ±SDs or *n* (%). Cut-offs for quintiles of red and processed meat intake were 32.00; 59.82; 86.81 and 122.14 g/d in men and 18.21; 39.73; 60.00 and 87.68 g/d in women.
[2]Among first-degree relatives.
[3]Missing for 7,842 (12.76%) subjects.

and vegetable intake, to have a lower educational level and to be less physically active.

Associations between red and processed meat intakes and overall, breast and prostate cancer risk are presented in Table 2. Red meat intake was associated with increased overall cancer risk (HR $_{Q5vs.Q1} = 1.31$; 95% CI 1.10, 1.55; $p_{trend} = 0.01$) and increased breast cancer risk (HR $_{Q5.Q1} = 1.83$; 95% CI 1.33, 2.51; $p_{trend} = 0.002$), but not with prostate cancer risk ($p_{trend} = 0.9$). This association between red meat intake and increased breast cancer risk was observed in both premenopausal ($HR_{Q5vs.Q1} = 2.04$ (1.03–4.06)) and postmenopausal women [$HR_{Q5vs.Q1} = 1.79$ (1.26–2.55); Table 3], and was similarly observed when analyses excluded cases diagnosed during their first year of follow-up [413 cases/40,892 non-cases included; $HR_{Q5vs.Q1} = 1.82$ (1.27, 2.62)] or when analyses were restricted to invasive breast cancers [470 cases/45,386 non-cases; $HR_{Q5vs.Q1} = 1.78$ (1.26, 2.50); data not tabulated. Results with and without BMI adjustment were very similar for overall, breast and prostate cancers models (without BMI adjustment in Supporting Information Table 2). No association was detected for processed meat intake ($p_{trend} = 0.5$, 0.4 and 0.3 for overall, breast and prostate cancers, respectively,

Table 2. Associations between quintiles of red and processed meat intake and overall, breast, and prostate cancer risk, from multivariable Cox proportional hazard models, NutriNet-Santé cohort, France, 2009–2016 ($n = 61,476$)[1]

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	*p*-trend
Red meat						
All cancers						0.01
N for cases/non-cases	233/12,101	359/11,898	307/12,001	358/11,876	352/11,991	
Multivariable HR (95%CI)	1	1.24 (1.05, 1.47)	1.06 (0.89, 1.26)	1.22 (1.03, 1.45)	1.31 (1.10, 1.55)	
Breast cancer						0.002
N for cases/non-cases	59/9,160	124/9,030	114/9,076	123/9,010	124/9,110	
Multivariable HR (95%CI)	1	1.68 (1.23, 2.31)	1.58 (1.14, 2.17)	1.70 (1.24, 2.34)	1.83 (1.33, 2.51)	
Prostate cancer						0.9
N for cases/non-cases	28/3,087	66/3,037	33/3,085	54/3,047	41/3,068	
Multivariable HR (95%CI)	1	1.70 (1.09, 2. 68)	0.87 (0.52, 1.45)	1.38 (0.86, 2.20)	1.28 (0.78, 2.11)	
Processed meat						
All cancers						0.5
N for cases/non-cases	403/17,148	221/6,830	350/1,1929	351/11,949	284/12,011	
Multivariable HR (95%CI)	1	1.08 (0.91, 1.28)	1.03 (0.88, 1.19)	1.05 (0.90, 1.22)	0.93 (0.79, 1.10)	
Breast cancer						0.4
N for cases/non-cases	133/13,809	63/4,380	113/9,055	134/9,057	101/9,085	
Multivariable HR (95%CI)	1	1.19 (0.88, 1.62)	1.08 (0.83, 1.39)	1.28 (1.00, 1.64)	1.05 (0.80, 1.38)	
Prostate cancer						0.3
N for cases/non-cases	37/3,572	42/2,566	57/3,054	45/3,064	41/3,068	
Multivariable HR (95%CI)	1	1.21 (0.77, 1.91)	1.39 (0.91, 2.13)	1.17 (0.74, 1.84)	1.35 (0.84, 2.20)	
Red and processed meat						
All cancers						0.3
N for cases/non-cases	266/12,026	339/11,959	342/11,961	344/11,943	318/11,978	
Multivariable HR (95%CI)	1	1.11 (0.94, 1.30)	1.08 (0.91, 1.27)	1.10 (0.93, 1.30)	1.12 (0.94, 1.33)	
Breast cancer						0.05
N for cases/non-cases	80/9,105	101/9,086	128/9,065	126/9,053	109/9,077	
Multivariable HR (95%CI)	1	1.10 (0.82, 1.49)	1.35 (1.02, 1.81)	1.36 (1.02, 1.81)	1.26 (0.93, 1.71)	
Prostate cancer						0.8
N for cases/non-cases	37/3,070	48/3,063	54/3,056	42/3,066	41/3,069	
Multivariable HR (95%CI)	1	1.07 (0.69, 1.65)	1.21 (0.79, 1.85)	0.93 (0.59, 1.48)	1.17 (0.72, 1.89)	

Sex-specific cut-offs for quintiles of red meat intake were 12.59; 37.14; 57.15 and 86.75 g/d in men and 0.14; 24.67; 42.15 and 65.71 g/d in women.
Sex-specific cut-offs for quintiles of processed meat intake were 0.20; 11.61; 25.45 and 45.86 g/d in men and 0.06; 5.36; 14.64 and 29.00 g/d in women.
Sex-specific cut-offs for quintiles of red and processed meat intake were 32.00; 59.82; 86.81 and 122.14 g/d in men and 18.21; 39.73; 60.00 and 87.68 g/d in women.
CI, confidence interval, HR, Hazard ratio.
[1]Multivariable models were adjusted for age (timescale), sex, energy intake without alcohol, number of 24 hrs-dietary records, smoking status, educational level, physical activity, height, BMI, alcohol intake, family history of cancers, lipids intake, fruits, vegetables, menopausal status and number of children (breast cancer models), red meat intake (where processed meat was analyzed) and processed meat intake (where red meat was analyzed).

Table 2). No association was statistically significant for red or processed meat intake with colorectal or with lung cancers or with lymphomas (Supporting Information Table 1). No interaction was detected between red or processed meat intake and fruit and vegetable or individual antioxidant intakes (vitamins C, E, beta-carotene and selenium) regarding overall and site-specific cancer risk (all $p > 0.05$, data not shown).

Discussion

In this large prospective cohort, red meat intake was significantly associated with increased overall and breast cancer risks. No association was observed for prostate cancer. Processed meat intake was not associated with cancer risk in this study.

For red meat, our result of a direct association with breast cancer risk is consistent with two recent meta-analyses: Guo

Table 3. Associations between quintiles of red and processed meat intake and breast cancer risk according to menopausal status from multivariable Cox proportional hazards models, NutriNet-Santé cohort, France, 2009–2016 ($n = 46,474$)[1]

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	*p*-trend
Red meat						
Pre-menopausal breast cancer						0.4
N for cases/non-cases	12/4,732	50/4,502	36/4,618	43/4,608	28/4,622	
Multivariable HR (95%CI)	1	3.36 (1.77, 6.38)	2.37 (1.22, 4.60)	2.91 (1.52, 5.57)	2.04 (1.03, 4.06)	
Post-menopausal breast cancer						0.002
N for cases/non-cases	48/5,347	73/5,307	81/5,308	78/5,319	95/5,281	
Multivariable HR (95%CI)	1	1.28 (0.88, 1.86)	1.46 (1.02, 2.09)	1.40 (0.97, 2.01)	1.79 (1.26, 2.55)	
Processed meat						
Pre-menopausal breast cancer						0.5
N for cases/non-cases	32/6,591	28/2,645	32/4,619	40/4,614	37/4,613	
Multivariable HR (95%CI)	1	1.62 (0.96, 2. 73)	1.09 (0.66, 1.80)	1.34 (0.83, 2.17)	1.30 (0.79, 2.15)	
Post-menopausal breast cancer						0.7
N for cases/non-cases	101/8,309	36/2,327	79/5,299	93/5,306	66/5,321	
Multivariable HR (95%CI)	1	1.08 (0.73, 1.60)	1.07 (0.79, 1.44)	1.28 (0.95, 1.72)	0.95 (0.69, 1.32)	
Red and processed meat						
Pre-menopausal breast cancer						0.8
N for cases/non-cases	23/4,609	36/4,632	41/4,612	40/4,608	29/4,621	
Multivariable HR (95%CI)	1	1.29 (0.76, 2.19)	1.40 (0.83, 2.36)	1.40 (0.83, 2.37)	1.05 (0.59, 1.86)	
Post-menopausal breast cancer						0.02
N for cases/non-cases	57/5,331	66/5,304	80/5,325	88/5,299	84/5,303	
Multivariable HR (95%CI)	1	1.06 (0.74, 1.52)	1.26 (0. 90, 1.77)	1.40 (0.99, 1.96)	1.41 (0.99, 2.01)	

In premenopausal women: cut-offs for quintiles of red meat intake were 0.29; 24.00; 42.14; 67.7 g/d; cut-offs for quintiles of processed meat intake were 0.11; 6.79; 16.43; 31.89 g/d; cut-offs for quintiles of red and processed meat intake were 18.57; 40.40; 61.79; 91.16 g/d.
In postmenopausal women: cut-offs for quintiles of red meat intake were 2.68; 25.37; 42.68; 65.00 g/d; cut-offs for quintiles of processed meat intake were 0.06; 5.14; 14.29; 27.26 g/d; cut-offs for quintiles of red and processed meat intake were 18.21; 39.29; 58.79; 85.06 g/d..
CI: confidence interval, HR: Hazard ratio.
[1]Multivariable models were adjusted for age (timescale), energy intake without alcohol, number of 24 hrs-dietary records, smoking status, educational level, physical activity, height, BMI, alcohol intake, family history of cancers, lipids intake, fruits, vegetables, hormone replacement therapy (for postmenopausal group), number of children, contraception (for premenopausal group), red meat intake (where processed meat was analyzed) and processed meat intake (where red meat was analyzed).

et al.[14] based on 14 cohort studies for red meat and 12 cohort studies for processed meat, and Wu *et al.*[15] based on 12 cohort studies for red meat and 15 cohort studies for processed meat, both showing positive associations with breast cancer risk. The two prospective studies published after this meta-analysis also suggest direct associations between red meat intake and post-menopausal breast cancer risk in the NIH-AARP cohort[16] and increased breast density.[17] In a previous study performed on the SU.VI.MAX cohort, we did not observe statistically significant relationships between red meat and breast cancer risk. However, red meat intakes in women of the SU.VI.MAX cohort were relatively low (fourth quartile <500 g/week), while they were higher in the present NutriNet-Santé cohort, where 19.60% exceeded 500 g of red meat per week. In the French general population, about one out of four adults consume >500 g/week of red meat.[3] In Europe the median range of daily red meat intake is 24–57 g/day,[36] while mean intake is about 53 g/d in the U.S.[37]

Regarding prostate cancer, our null result is consistent with two large and recent meta-analyses of prospective studies, performed by the WCRF/AICR in 2014[18] and Blysma *et al.* in 2015.[19] In a pooled analysis of 15 cohort studies, Wu *et al.*[20] did not observe any association between red meat intake and overall prostate cancer risk, but showed a modest positive association for tumors identified as advanced stage at diagnosis. In our study, our results did not differ according to Gleason score (< or ≥7) [data not shown]. However, statistical power was limited for this sub-analysis. In the WCRF/AICR meta-analyses, the summary RR were not statistically significant for the different prostate cancer subtypes, (RR per 100 g/d = 0.99 (0.89, 1.11) for advanced/high grade and 1.19 (0.88, 1.59) for fatal cases).[18]

The small number of cancer cases other than breast and prostate locations did not allow us to have enough statistical analysis to conclude for these locations. However, the pro-carcinogenic effect of high red meat intake on colorectal carcinogenesis has been well established in several national and

international collective expert evaluations.[1,3,4] In 2012, the WCRF/AICR also judged the direct association between red meat intake and pancreatic cancer risk as "suggestive". Along with the positive association observed for breast cancer, these may contribute to explain the positive association observed in the present study between red meat intake and overall cancer risk. It is also possible that the lack of association with processed meat might be a chance finding or could change with longer follow-up.

While several studies suggested direct associations between processed meat intake and colorectal,[1,3,4] breast,[13,14,16] stomach,[38] or pancreatic[39] cancer risk, no association was detected in the present study. This may be explained by the fact that processed meat intakes were too low to properly investigate any adverse effect. Indeed, the cut-off for quintile 5 of processed meat intake was 45.9 g/d for men and 29.0 g/d for women, that is, lower than the 50 g/d upper dose recommended by the WCRF/AICR for colorectal cancer prevention.[4] In the French general population, more than one out of four adults consume at least 50 g of processed meat per Day.[3] In Europe the median range of daily processed meat intake is 5–49 g/Day,[36] while mean intake is about 18 g/d in the U.S.[37]

Our epidemiological findings are supported by mechanistic data. Red and processed meat contain pro-carcinogenic components, such as heterocyclic aromatic amines (HAA), polycyclic aromatic hydrocarbons (PAHs) resulting from meat processing or preparation (such as cooking at high-temperature), nitrites (used as additives) and induces N-nitroso compounds (NOCs) formation in the digestive tract.[40–44] These chemicals may exert a pro-carcinogenic effect through direct DNA damage and have been associated with mammary tumor development in animal[7,9,41] and human[8,10,45] studies.[13,46,47] Most importantly, red meat contains high levels of heme iron, which may contribute to initiate carcinogenesis via several mechanisms, including the production of genotoxic free radicals, NOCs or through lipid peroxidation.[5,48–50]

Strengths of this study include its prospective design, its large sample size, and the assessment of usual dietary intakes using repeated 24 hrs-dietary records based on a recent food composition database with a large choice of items (>3,300). These repeated 24 hrs-dietary records allowed a better insight into the food products consumed compared to food frequency questionnaires with more aggregated food items. However, some limitations should be acknowledged. First, caution is needed regarding the extrapolation of these results since this study included volunteers involved in a long-term cohort study investigating the association between nutrition and health, with overall more health-conscious behaviors and higher professional and/or educational level compared to the general French population. Thus, unhealthy dietary behaviors may have been underrepresented in this study, which may have weakened the observed associations and may have prevented us from observing significant associations for processed meat. Second, although the number of overall cancer cases was reasonably large, the number of cancers at any given site was more restricted, which did not allow us to investigate more cancers sites and receptor types for breast cancer. Finally, the observed relationships could be partly affected by unmeasured or residual confounding. However, main potential confounders have been accounted for in this study; thus, it is unlikely that residual confounding entirely explains the observed associations.

In conclusion, this prospective cohort study brings new contribution into the role of red and processed meat intake as cancer risk factors. We observed that red meat intake was associated with increased overall and breast cancer risk, in line with mechanistic hypotheses from experimental studies. If confirmed, these findings suggest that limiting red meat intake may not only be beneficial for colorectal cancer, but also for the prevention of other tumor locations such as breast cancer.

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The author's responsibilities were as follows – A.D. and M.T.: designed the research; S.H., M.T., P.G.: conducted the research; A.D.: performed statistical analysis and wrote the paper; M.T.: supervised the study; M.D., P.L.M., P.G., S.H., P.F., B.A., F.G., F.H.P.: contributed to the data interpretation and revised each draft for important intellectual content; and MT had primary responsibility for final content. All authors read and approved the final manuscript. The funders had no role in the design, implementation, analysis, or interpretation of the data. The authors have no conflict of interest to disclose.

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Original Research

Red and processed meat consumption and breast cancer: UK Biobank cohort study and meta-analysis

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Abstract ***Aim:*** Red and processed meat may be risk factors for breast cancer due to their iron content, administration of oestrogens to cattle or mutagens created during cooking. We studied the associations in UK Biobank and then included the results in a meta-analysis of published cohort studies.
Methods: UK Biobank, a general population cohort study, recruited participants aged 40–69 years. Incident breast cancer was ascertained via linkage to routine hospital admission, cancer registry and death certificate data. Univariate and multivariable Cox proportional hazard models were used to explore the associations between red and processed meat consumption and breast cancer. Previously published cohort studies were identified from a systematic review using PubMed and Ovid and a meta-analysis conducted using a random effects model.
Results: Over a median of 7 years follow-up, 4819 of the 262,195 women developed breast cancer. The risk was increased in the highest tertile (>9 g/day) of processed meat consumption (adjusted hazard ratio [HR] 1.21, 95% confidence interval [CI] 1.08–1.35, p = 0.001). Collation with 10 previous cohort studies provided data on 40,257 incident breast cancers in 1.65 million women. On meta-analysis, processed meat consumption was associated with overall (relative risk [RR] 1.06, 95% CI 1.01–1.11) and post-menopausal (RR 1.09, 95% CI 1.03–1.15), but not pre-menopausal (RR 0.99, 95% CI 0.88–1.10), breast cancer. In UK Biobank and the meta-analysis, red meat consumption was not associated with breast cancer (adjusted HR 0.99 95% CI 0.88–1.12 and RR 1.03, 95% CI 0.99–1.08, respectively).

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Conclusions: Consumption of processed meat, but not red meat, may increase the risk of breast cancer.

1. Introduction

In the United Kingdom, 1 in 8 women will develop breast cancer [1], but more than one-quarter of cases could be prevented by reduced exposure to exogenous oestrogens, reduced obesity, increased physical activity and breastfeeding [1]. There is a lack of consensus on whether red and processed meat consumption is a risk factor for breast cancer [2]. Four meta-analyses have produced conflicting results [3–6] due to wide inclusion criteria, resulting in the inclusion of very heterogeneous studies. We studied whether red and processed meat consumption were associated with the risk of breast cancer in UK Biobank; then included the results in a meta-analysis of prospective cohort studies using rigorous inclusion criteria.

2. Materials and methods

2.1. UK Biobank

UK Biobank recruited 273,466 women aged 40–69 years from the general population between 2007 and 2010. Baseline socioeconomic and lifestyle information were collected via a self-completed, touch-screen questionnaire and anthropometric measurements taken by trained staff. Self-reported moderate and vigorous physical activity were converted to METs·min·week^{-1}, and dichotomised to inactive (<600 METs·min·week^{-1}) and active (≥600 METs·min·week^{-1}). Dietary information was collected using a self-completed food frequency questionnaire. Frequency of beef, pork and lamb intake (excluding processed meat) and frequency of processed meat intake were recorded. These were converted into probabilities of daily consumption, multiplied by normal portion sizes [7] and then weighted by size of portion: small 0.5, medium 1.0 or large 1.5. We then derived four categories of red/processed meat intake: zero intake and tertiles of consumption for those consuming some. Follow-up information (min 5.33 years and max 9.89 years) on the date of first diagnosis of cancer was obtained via linkage to three routine administrative databases: cancer registrations, death certificates and hospital admissions. Date and cause of death were obtained from death certificates held by the National Health Service (NHS) Information Centre (England and Wales) and the NHS Central Register Scotland (Scotland). Date and cause of hospital admissions were obtained from the Health Episode Statistics (HES) for England and Wales and the Scottish Morbidity Record 01 (SMR01) for Scotland. At the time of analysis, mortality data were available up to 31 January 2016 and hospital admission and cancer registry data until 31 March 2015. Therefore, follow-up was censored at 31 January 2016 or date of death if this occurred earlier. There were 54 participants who withdrew consent from UK Biobank at the time of analysis. All databases used the International Classification of Diseases and we defined breast cancer as ICD10 code C50.

We excluded women with a record of breast cancer at baseline. Cox proportional hazard models were used to examine the associations between red/processed meat consumption and breast cancer using zero consumption as the referent category. We ran four incremental models for each: univariate, multivariable adjusted for sociodemographic factors (age, sex, ethnic group and deprivation index); multivariable also adjusted for lifestyle factors (smoking status, frequency of alcohol consumption, body mass index and physical activity) and multivariable also adjusted for potential dietary confounders (cooked vegetables, raw vegetables and type of bread). We tested for statistical interactions and, where significant, subgroup analyses were undertaken. All analyses were repeated after stratifying women into pre- and postmenopausal subgroups. In the latter, we included the use of hormone replacement therapy as a covariate in the fully adjusted model. We also conducted landmark analyses, excluding the first 2 years of follow-up. This study was performed under generic ethical approval obtained by UK Biobank from the NHS National Research Ethics Service (ref 11/NW/0382, 17 June 2011). All analyses were undertaken using Stata, version 14.

2.2. Meta-analysis

Two authors (JJA and NDMD) searched PubMed and Ovid using the search term breast cancer combined with meat, red meat, processed meat, preserved meat, pork, beef, veal, mutton, lamb, ham, sausage or bacon; consistent with the most recently published meta-analysis [6]. However, inclusion was restricted to prospective, general population cohort studies. We excluded case-control studies and studies that measured only beef intake. Where more than one study was conducted on the same cohort, only the most recent was included. The last search was conducted on 15 January 2017. Meta-analysis was undertaken using a random effects model; stratified by type of meat (red and processed) and outcome (pre-,

Table 1
Demographic, lifestyle and dietary characteristics of female UK Biobank participants according to whether or not they developed breast cancer.

Characteristics	No breast cancer	Breast cancer	P value*
	N = 257,376	N = 4819	
	N (%)	N (%)	
Ethnic group			
White	242,024 (94.5)	4621 (96.3)	<0.001
Asian	4414 (1.7)	58 (1.2)	
Black	4519 (1.8)	42 (0.9)	
Other	5206 (2.0)	76 (1.6)	
Missing	1213	22	
Smoking status			
Never	153,066 (59.8)	2774 (54.8)	0.002
Former	79,787 (31.2)	1609 (33.6)	
Current	23,090 (9.0)	413 (8.6)	
Missing	1433	23	
Physical activity			
Inactive	124,496 (48.4)	2440 (50.6)	0.002
Active	132,880 (51.6)	2379 (49.4)	
Missing	0	0	
Alcohol frequency			
Never	41,104 (16.0)	905 (16.7)	0.017
Special occasions	52,595 (20.5)	1069 (22.2)	
1–2/month	66,088 (25.8)	1209 (25.2)	
1–2/week	33,561 (13.1)	608 (12.7)	
3–4/week	38,734 (15.1)	686 (14.3)	
Daily	24,586 (9.6)	431 (9.0)	
Missing	708	11	
Cooked vegetables (spoons/day)			
0	6444 (2.5)	117 (3.0)	0.528
1	35,749 (14.1)	663 (13.9)	
2	87,239 (34.3)	1645 (34.5)	
3	72,735 (28.6)	1361 (28.5)	
4	28,167 (11.1)	563 (11.8)	
≥5	24,133 (9.5)	425 (8.9)	
Missing	2909	45	
Raw vegetables (spoons/day)			
0	18,097 (7.1)	385 (8.1)	0.048
1	80,302 (31.6)	1545 (32.3)	
2	64,216 (25.3)	1187 (24.9)	
3	40,895 (16.1)	766 (16.0)	
4	21,855 (8.6)	398 (8.3)	
≥5	28,839 (11.3)	496 (10.4)	
Missing	3172	42	
Bread			
Brown/wholemeal	178,913 (73.8)	3374 (73.9)	0.828
White/other	63,570 (26.2)	1190 (26.1)	
Missing	14,893	255	
Red meat (g/day)			
0	22,059 (8.7)	376 (7.9)	<0.001
1–19	116,675 (45.9)	2069 (43.6)	
19–25	42,869 (16.7)	858 (18.1)	
>25	72,539 (28.5)	1477 (30.5)	
Missing	3234	69	
Processed meat (g/day)			
0	32,456 (12.7)	521 (10.9)	0.023
1–4	99,269 (38.7)	1875 (39.1)	
5–9	70,313 (27.4)	1393 (29.0)	
>9	54,268 (21.2)	1011 (21.1)	
Missing	1070	19	
	Mean (SD)	Mean (SD)	
Age	56.2 (8.0)	57.6 (7.6)	
Missing	0	0	
Deprivation index	−1.3 (3.1)	−1.5 (3.0)	<0.001
Missing	313	3	
Body mass index (kg/m^2)	27.1 (5.2)	27.6 (5.1)	<0.001
Missing	4818	87	

*t-test for age, BMI and fibre intake; Mann–Whitney U test for deprivation index; chi-squared test for sex, ethnic group and type of bread; chi-squared test for trend for smoking and intake of alcohol, meat and vegetables.
N, number; BMI, body mass index; SD, standard deviation.

post-menopausal and overall breast cancer). We performed Egger's and Begg's tests and used funnel plots to assess potential bias. Heterogeneity between the studies was tested using the I-squared statistic. All analyses were undertaken using Stata, version 14.

3. Results

3.1. UK Biobank

Of the 273,466 female participants, 262,195 had no record of breast cancer at baseline and, therefore, were eligible for inclusion. Of these, 4819 (1.8%) developed incident breast cancer over a median follow-up period of 7 years (interquartile range, IQR 6.3–7.7). The participants who developed breast cancer were older, more affluent, less physically active, more likely to be white and former smokers, had higher body mass indices and reported lower alcohol and raw vegetable intake, but higher intake of red and processed meat (Table 1).

Overall, 3303 (1.3%) of the 262,195 women had missing data on consumption of red meat; of the remainder, 22,435 (8.6%) consumed no red meat, 118,744 (45.3%) consumed <19 g/day, 43,727 (16.7%) 19–25 g/day and 73,986 (28.2%) >25 g/day. In relation to processed meat consumption, 1089 (0.4%) had missing data and, of the remainder, 32,977 (12.6%) consumed none, 101,144 (38.6%) <4 g/day, 71,706 (27.4%) 4–9 g/day and 55,279 (21.1%) >9 g/day.

In the univariate Cox proportional hazards model, there was a significant overall association between red meat consumption and risk of incident breast cancer (Table 2). Adjustment for potential sociodemographic confounders attenuated the overall association and, following further adjustment for potential lifestyle and dietary confounders, it was no longer statistically significant. There were no significant interactions with any of the covariates or menopausal status. On subgroup analyses, the associations between red meat consumption and breast cancer were not significant in either pre- or post-menopausal women. Landmark analyses, excluding the first 2 years of follow-up, did not alter the results. Supplementary Table 1 contains the results re-run using the lowest tertile of red meat intake as the referent category.

Table 2
Cox proportional hazard models of the risk of breast cancer associated with red and processed meat consumption.

Intake	Univariate			Multivariable*			Multivariable**			Multivariable***		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Red meat (g/day)												
		N = 258,892	0.008		N = 257,876	0.177		N = 252,309	0.622		N = 235,233	0.431
0	1			1			1			1		
<19	1.04	0.93–1.16	0.511	0.99	0.89–1.11	0.885	0.96	0.86–1.08	0.528	0.96	0.85–1.08	0.469
19–25	1.12	1.00–1.27	0.064	1.06	0.93–1.12	0.395	1.02	0.90–1.16	0.747	1.02	0.90–1.16	0.738
>25	1.12	1.00–1.25	0.058	1.04	0.93–1.16	0.537	0.99	0.88–1.11	0.828	0.99	0.88–1.12	0.914
Processed meat (g/day)												
		N = 261,106	0.001		N = 260,064	<0.001		N = 254,356	0.002		N = 236,876	0.005
0	1			1			1			1		
<4	1.17	1.07–1.30	0.001	1.15	1.05–1.28	0.003	1.15	1.04–1.27	0.006	1.15	1.04–1.28	0.007
4–9	1.22	1.11–1.35	<0.001	1.21	1.09–1.33	<0.001	1.19	1.07–1.32	0.001	1.19	1.07–1.33	0.002
>9	1.22	1.10–1.36	<0.001	1.23	1.10–1.36	<0.001	1.21	1.09–1.35	0.001	1.21	1.08–1.35	0.001

*Adjusted for age, deprivation and ethnic group, **also adjusted for smoking, alcohol, body mass index and physical activity, ***also adjusted for consumption of cooked and raw vegetables and type of bread; HR, hazard ratio; CI, confidence interval; N, number.

In the univariate analysis, there was a statistically significant dose–response relationship between processed meat consumption and breast cancer (Table 2). Adjustment for potential sociodemographic, lifestyle and dietary confounders did not attenuate the results. There was a significant dose–response relationship across the tertiles of processed meat consumption; whereby, participants in the low, medium and highest tertiles of consumption remained significantly more likely to develop breast cancer than those with zero intake. In the fully adjusted model the results were as follows: <4 g/day hazard ratio (HR) 1.15, 95% confidence interval (CI) 1.04–1.28, p = 0.007; 4–9 g/day HR 1.19, 95% CI 1.07–1.33, p = 0.002; >9 g/day HR 1.21, 95% CI 1.08–1.35, p = 0.001 (overall p_{trend} = 0.005). There was a statistically significant interaction with the intake of cooked vegetables (p = 0.009). There was a weaker association between processed meat intake and breast cancer among participants with the lowest intake of cooked vegetable. This was due to the absolute risk already being higher in this subgroup; among participants who ate no processed meat, the incidence of breast cancer was 2.46 per 1000 population per annum among those with low intake of cooked vegetables compared with only 2.01 per 1000 per annum among those with high vegetable intake. Among participants who had the highest intake of processed meat, the incidence of breast cancer was 2.55 per 1000 population per annum among those with low cooked vegetable intake and 2.35 per 1000 per annum among those with high intake. There was no significant interaction with menopausal status. However, in the subgroup of pre-menopausal women, the increased risk of breast cancer only reached statistical significance in the highest tertile of processed meat intake (fully adjusted model: <4 g/day HR 1.24, 95% CI 0.98–1.57, p = 0.069; 4–9 g/day HR 1.21, 95% CI 0.95–1.54, p = 0.131; >9 g/day HR 1.32, 95% CI 1.03–1.69, p = 0.032). Among post-menopausal women, the risk of breast cancer was significantly higher among all groups that consumed processed meat (fully adjusted model: <4 g/day HR 1.16, 95% CI 1.03–1.31, p = 0.016; 4–9 g/day HR 1.20, 95% CI 1.05–1.36, p = 0.006; >9 g/day HR 1.20, 95% CI 1.05–1.37, p = 0.008). In the landmark analyses, excluding the first 2 years of follow-up, the effect estimates remained unaffected (fully adjusted model: <4 g/day HR 1.15, 95% CI 1.02–1.29, p = 0.022; 4–9 g/day HR 1.19, 95% CI 1.05–1.34, p = 0.006; >9 g/day HR 1.23, 95% CI 1.08–1.39, p = 0.002). Supplementary Table 1 shows the results re-run using the first tertile of processed meat consumption as the reference category.

3.2. Meta-analysis

A total of 124 and 84 publications were identified by searching the PubMed and Ovid databases, respectively, of which 78 were excluded as duplicates. The remaining 130 articles were screened, together with nine additional publications identified from reference lists. Of these, 122 were excluded because they did not satisfy the inclusion criteria. A further five studies were excluded due to repeat analyses conducted on the same cohort and two due to inadequate exposure information; resulting in 10 eligible cohort studies in addition to UK Biobank (Fig. 1). The 10 previous studies comprised a total of 35,438 incident cancers occurring in 1,386,799 women [8–17]. Combined with UK Biobank, this produced a total of 11 studies with data on 40,257 incident cancers in 1,648,994 women (Table 3).

Of the 11 cohort studies, 10 reported the association between red meat consumption and overall risk of breast cancer; of these, six also reported results separately for pre- and post-menopausal breast cancer. The 11th study only examined the association with post-menopausal breast cancer (Table 3). The 10 studies produced a pooled relative risk (RR) for breast cancer, overall, of 1.03 (95% CI 0.99–1.08) (Fig. 2). The funnel plot was reasonably symmetrical with one small study outlier (Supplementary Fig. 1a), and both Begg's

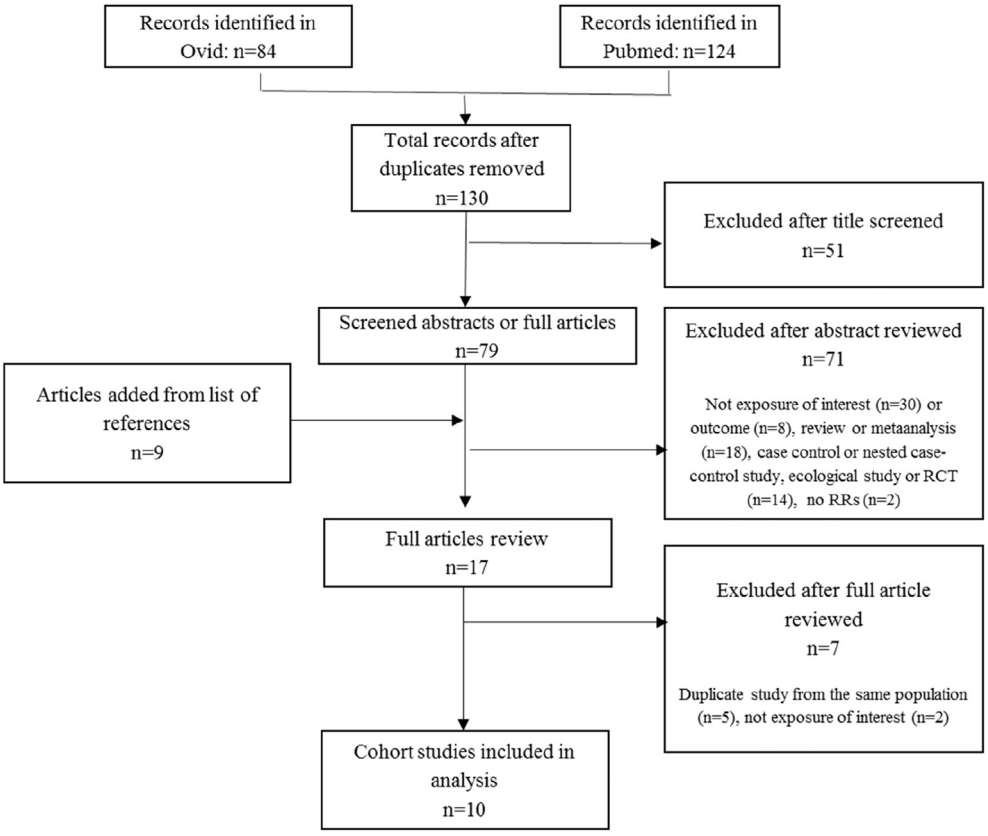

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of study selection process (Presented according to PRISMA guidelines).

(p = 0.210) and Egger's (p = 0.317) tests were non-significant. Overall there was a medium-level heterogeneity (I^2 44.0%) that was not statistically significant (p = 0.065).

The six studies on pre-menopausal breast cancer produced a pooled RR for high consumption of red meat of 1.02 (95% CI 0.92–1.11) (Fig. 2). Both Begg's (p = 0.573) and Egger's (p = 0.272) tests were non-significant indicating no significant publication bias, and the funnel plot was symmetrical with no study outliers (Supplementary Fig. 1b). The level of heterogeneity was low (I^2 0.0%) and not statistically significant (p = 0.530). The pooled RR for post-menopausal breast cancer, from the six relevant studies, was 1.03 (95% CI 0.97–1.08) (Fig. 2). Both Begg's (p = 0.764) and Egger's (p = 0.483) tests were non-significant, and the funnel plot was symmetrical with one small study outlier (Supplementary Fig. 1c). Overall there was low heterogeneity (I^2 34.6%) that was not statistically significant (p = 0.177).

Of the nine cohort studies on processed meat consumption, eight examined the association with overall risk of breast cancer (Fig. 3); five of these also studied both pre- and post-menopausal breast cancer. The ninth study reported results for post-menopausal breast cancer only. For overall risk of breast cancer, the pooled RR from the eight studies was 1.06 (95% CI 1.01–1.11) (Fig. 3). The funnel plot was reasonably symmetrical with one small study outlier (Supplementary Fig. 2a). Both Egger's (p = 0.141) and Begg's (p = 0.108) tests were non-significant indicating no significant publication bias. Overall there was a medium-level heterogeneity (I^2 61.5%) that was statistically significant (p = 0.011).

The pooled RR for pre-menopausal breast cancer, from the five relevant studies, was 0.99 (95% CI 0.88–1.10) (Fig. 3). Both Begg's (p = 1.000) and Egger's (p = 0.662) tests were non-significant, and the funnel plot was symmetrical with one small study outlier (Supplementary Fig. 2b). The level of heterogeneity was medium (I^2 39.5%) and not statistically significant (p = 0.158). The six relevant studies produced a pooled RR for post-menopausal breast cancer of 1.09 (95% CI 1.03–1.15) (Fig. 3). Both Begg's (p = 0.348) and Egger's (p = 0.570) tests were non-significant, and the funnel plot was symmetrical with one small study outlier (Supplementary Fig. 2c). Overall there was medium heterogeneity (I^2 40.2%) that was not statistically significant (p = 0.137).

4. Discussion

Among the 262,195 women in UK Biobank, those who consumed processed meat were at a higher risk of breast cancer; independent of sociodemographic, lifestyle, obesity and dietary factors included in this study. Our

Table 3
Characteristics of the cohort studies included in the meta-analysis.

Reference	Country	Cohort	Participants		Exposure details	Intake	Follow-up (years)	Breast cancer		Result		
			Age (years)	N				N	Type	RR	Lower CI	Upper CI
Holmes *et al.* (2003)												
	USA	NHS	30–55	88,647	Red meat	≥1.32 serving/day	18	4107	Overall	0.94	0.84	1.05
		NHS			Processed meat	≥0.46 serving/day			Overall	0.94	0.85	1.05
		NHS			Red meat				Post-menopausal	0.99	0.86	1.13
		NHS			Processed meat				Post-menopausal	1	0.88	1.13
		NHS			Red meat				Pre-menopausal	0.94	0.72	1.22
		NHS			Processed meat				Pre-menopausal	0.86	0.67	1.09
Cross *et al.* (2007)												
	USA	NIH AARP	50–71	494,036	Red meat	62.7 g/1,000 kcal	8.2	5872	Overall	1.02	0.93	1.12
		NIH AARP			Processed meat	22.6 g/1,000 kcal			Overall	1.03	0.94	1.12
Taylor *et al.* (2007)												
	UK	UK WCS	35–69	33,725	Red meat	>57 g/day	8	678	Overall	1.41	1.11	1.81
		UK WCS			Processed meat	>20 g/day			Overall	1.39	1.09	1.78
		UK WCS			Red meat				Post-menopausal	1.56	1.09	2.23
		UK WCS			Processed meat				Post-menopausal	1.64	1.14	2.37
		UK WCS			Red meat				Pre-menopausal	1.32	0.93	1.88
		UK WCS			Processed meat				Pre-menopausal	1.2	0.85	1.7
Ferucci *et al.* (2009)												
	USA	PLCOCST	55–74	52,158	Red meat	52.8 g/1,000 kcal	5.5	1205	Overall	1.23	1	1.51
		PLCOCST			Processed meat	16.9 g/1,000 kcal			Overall	1.12	0.92	1.36
Larsson *et al.* (2009)												
	Swedish	Swedish MC	40–71	61,433	Red meat	≥98 g/day	17.4	2952	Overall	0.98	0.86	1.12
Pala *et al.* (2009)												
	Europe	EPIC	25–75	319,826	Red meat	84.6 g/day	8.8	7119	Overall	1.06	0.98	1.14
					Processed meat	56.5 g/day			Overall	1.1	1	1.2
		EPIC			Red meat				Post-menopausal	1.05	0.94	1.18
					Processed meat				Post-menopausal	1.13	1	1.28
		EPIC			Red meat				Pre-menopausal	0.94	0.8	1.1
					Processed meat				Pre-menopausal	0.99	0.82	1.19
Genkinger *et al.* (2013)												
	USA	BWHS	21–69	52,062	Red meat	≥400 g/week	12	1268	Overall	1.02	0.83	1.24
					Processed meat	≥200 g/week			Overall	0.99	0.82	1.2
		BWHS			Red meat				Post-menopausal	0.86	0.62	1.2
					Processed meat				Post-menopausal	0.93	0.69	1.27
		BWHS			Red meat				Pre-menopausal	1.01	0.78	1.3
					Processed meat				Pre-menopausal	0.92	0.72	1.18
Farvid *et al.* (2014)												
	USA	NHSII	33–52	88,803	Red meat	1.50 serving/day	20	2830	Overall	1.22	1.06	1.4
		NHSII			Red meat				Post-menopausal	1.23	0.96	1.57
		NHSII			Red meat				Pre-menopausal	1.12	0.93	1.35
Pouchieu *et al.* (2014)												
	France	SUVIMAX		2367	Red meat	>63.7 g/day	11.3	102	Overall	1.01	0.58	1.74
		SUVIMAX			Processed meat	>43.5 g/day			Overall	2.46	1.28	4.72

Inoue-choi *et al.* (2016)											
USA	NIH AARP	62 (5.3)	193,742	Red meat	43.4 g/1,000 kcal	9.4	9305	Post-menopausal	1.03	0.96	1.11
	NIH AARP			Processed meat	14.5 g/1,000 kcal			Post-menopausal	1.09	1.01	1.17
Anderson *et al.* (2017)											
UK	UK Biobank	40–69	262,195	Red meat	37.8 g/day	7	4819	Overall	0.99	0.88	1.12
								Post-menopausal	0.95	0.82	1.1
								Pre-menopausal	1.09	0.85	1.4
				Processed meat	20.2 g/day			Overall	1.21	1.08	1.35
								Post-menopausal	1.2	1.05	1.37
								Pre-menopausal	1.32	1.03	1.69

N, number; RR, relative risk; CI, confidence interval; USA, United States of America; NHS, Nurses Health Study; NIH, National Institutes of Health; AARP, American Association for Retired Persons; UK, United Kingdom; WCS, Women's Cohort Study; PLCOCST, Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial; SU.VI.MAX, Supplementation en Vitamines et Mineraux Antioxydants; BWHS, Black Women's Health Study; EPIC, European Prospective Investigation into Cancer and Nutrition; Swedish MC, The Swedish Mammography Cohort. Referent category: Taylor *et al.* (2007) and Anderson *et al.* (2017) zero intake; otherwise zero/lowest intake (i.e. lowest intake category including zero intake).

results and the meta-analysis suggested the overall association is largely driven by the risk of post-menopausal breast cancer. Red meat consumption was not a risk factor for breast cancer in UK Biobank, after adjusting for confounding; nor in the meta-analysis.

A number of possible underlying mechanisms have been mooted [18]. Processed meat contains high levels of amines, and nitrate and nitrite are commonly added to enhance colour and flavour. All are precursors of N-nitroso compounds which are carcinogenic. The added nitrate together with the heme iron present in red meat enhances endogenous N-nitroso compound formation [17], whereas antioxidants inhibit it [19]. In a randomised controlled trial, consumption of processed meat (HR 2.46; 95% CI 1.28–4.72) and dietary heme (HR 2.80, 95% CI 1.42, 5.54) were both associated with breast cancer in the control arm, but not in the intervention arm which was given low-dose antioxidants [16,19]. A recent study has implicated the high content of N-glycolylneuraminic acid, an animal sugar, as a possible cause of chronic inflammation and tumour formation [20].

The mechanism most extensively studied has been the possible role of cooking. Cooking red meat can produce carcinogenic compounds such as heterocyclic amines and polycyclic aromatic hydrocarbons [21,22]. The likelihood of carcinogens being formed varies according to the method, temperature and duration of cooking. In a case-control study of 2386 women with breast cancer and 1703 healthy controls, there was an overall association between red meat consumption and breast cancer. However, on subgroup analysis the association was significant in women using high temperature cooking methods (odds ratio [OR] 1.5, 95% CI 1.3–1.9, $p < 0.001$) but not those using other cooking methods (OR 1.1, 95% CI 0.9–1.3, $p = 0.429$) [21]. A recent study found that high intake of smoked meats, that are high in polycyclic aromatic hydrocarbons, was associated with mortality from breast cancer [23].

Because the UK Biobank participants are not representative of the general population, summary statistics such as disease frequency cannot be generalised; however, estimates of effect size can [24]. Repeated 24-h dietary recall questionnaires are generally more accurate than food frequency questionnaires, but take longer to complete, and were only available on a minority of UK Biobank participants. Therefore, our study used data from the self-completed food frequency questionnaire; the usual methodology adopted in large-scale studies. To date, there has been no internal validation of the food frequency data within the UK Biobank population. Participants who completed the Oxford WebQ were more likely to be female, white, older, more affluent and better educated compared with the rest of the UK Biobank participants, which may have introduced response bias. Breast cancer was ascertained through a combination of hospital admission, cancer registry and death certificate data; therefore, it should be reasonably complete and

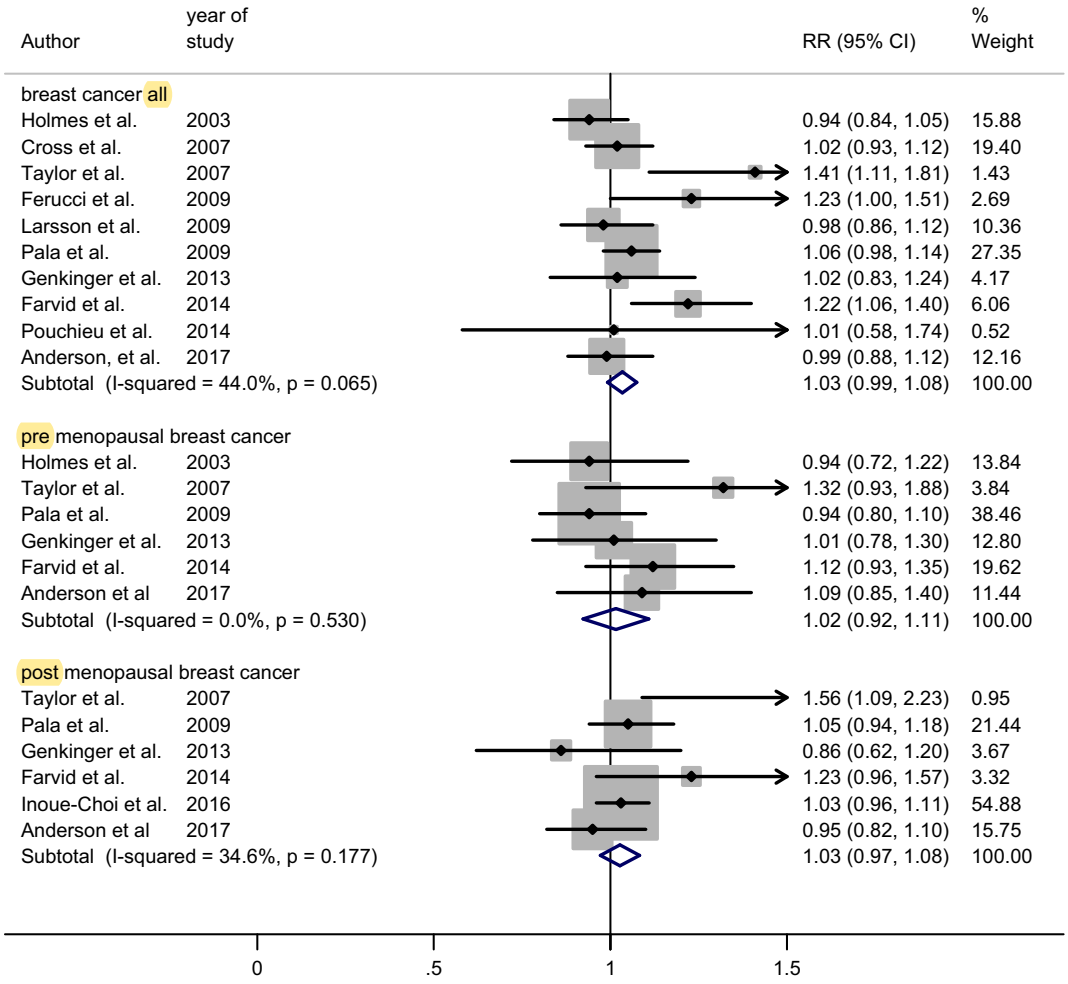

Fig. 2. Forest plot of cohort studies examining the association between red meat intake and breast cancer. RR, relative risk; CI, confidence interval.

selection bias unlikely. We were able to adjust for a wide range of confounders including sociodemographic, lifestyle and dietary factors; however, residual confounding is possible in any observational study. Although there was some evidence of a possible dose relationship, the largest increase in risk of breast cancer was between zero and low intake (4 g/day) of processed meat. Women who ate no processed meat may differ in other, unmeasured, ways or may have changed their diet as a result of ill-health. To check for potential reverse causation, we repeated the analyses using landmark analyses, and the results were similar. A limitation of our study was the inability to determine whether the associations varied according to the hormonal receptor status of tumours, due to lack of these data in UK Biobank. Our meta-analysis was the largest to date, including data on 40,257 incident cancers in over 1.6 million women from 11 independent cohorts. A limitation of our meta-analysis was the inconsistent approaches adopted by the individual studies in the number and range of confounders they included; therefore, we used a random effects approach to allow for differences in effect size between different study populations.

We obtained a similar pooled estimate as Guo *et al.* [6] for processed meat consumption but a non-significant pooled estimate for red meat consumption. The latter is due to our meta-analysis employing stricter inclusion criteria and methodology. We included only cohort studies, did not include duplicate information from repeat studies on the same cohort, included only estimates based on comparisons of the highest and lowest intake categories, excluded estimates based on increments in intake, included only evaluations of red meat and processed meat intake and excluded studies that analysed total meat consumption or only selected types of red meat, such as beef. In comparison, the most recently published meta-analysis, by Guo *et al.*, included three nested case-control studies [25–27] as well as cohort studies, and treated ORs as equivalent to RRs [26,27]. One of the nested case-control studies produced atypically high estimates of the associations, but these were derived from a study population with much higher levels of meat consumption in the highest category than our UK Biobank study. Guo *et al.* also included two studies that were undertaken on the same cohort as two

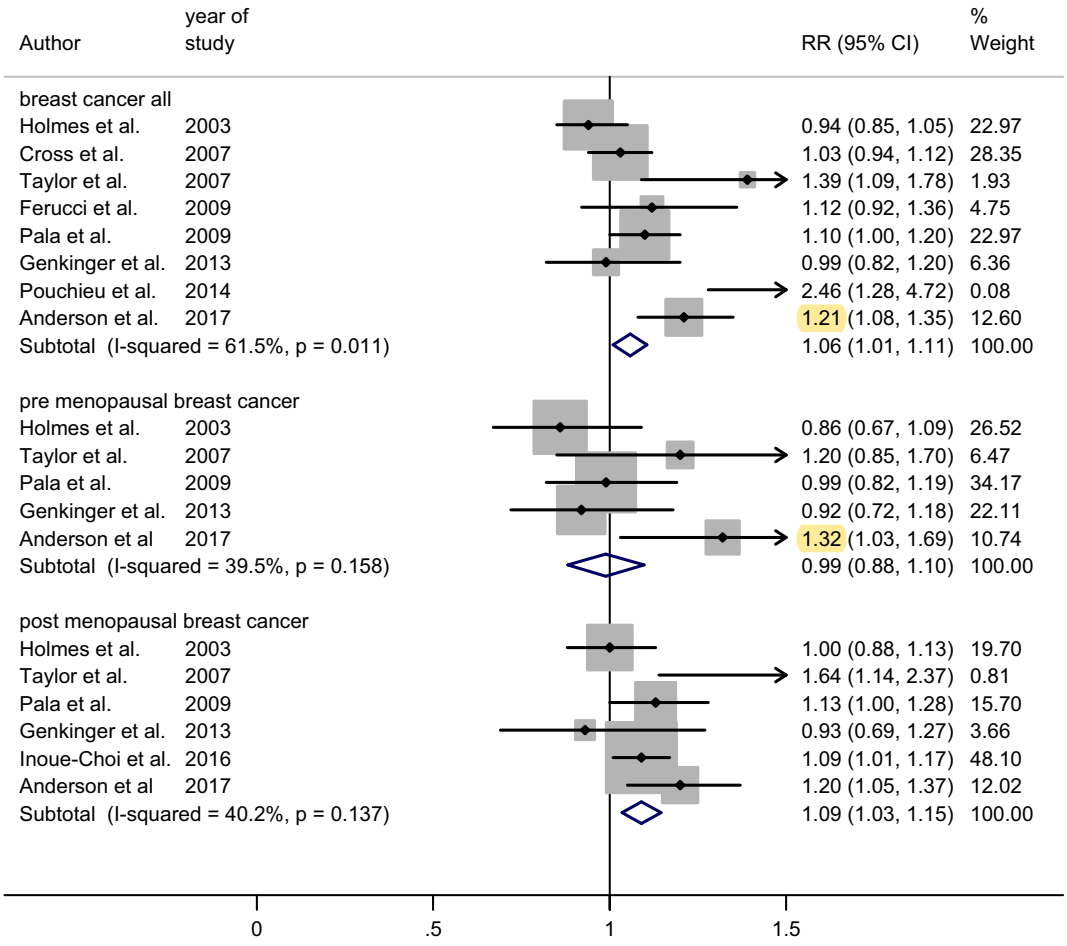

Fig. 3. Forest plot of cohort studies examining the association between processed meat intake and breast cancer. RR, relative risk; CI, confidence interval.

other included studies [15,28]. Furthermore, they included a study on 6156 women who participated in the National Health Epidemiologic Follow-up Study, which compared women according to beef, rather than total red meat, intake [29]. Therefore, the groups reporting no and low beef intake will have included women who consumed other forms of red meat; such as pork, lamb and game. Because of our tighter inclusion criteria, the heterogeneity of the studies included in our meta-analysis was lower than those included in the meta-analysis conducted by Guo *et al.*: I-square for red meat 44.0% versus 62.2%.

A previous meta-analysis based on estimates of incremental intake of red and processed meat conducted by the World Cancer Research fund reported similar findings to this study [30]. They found that there was no association between red meat intake and breast cancer, whereas the pooled RR for 50 g/day intake of processed meat and post-menopausal breast cancer was 1.13, 95% CI 0.99–1.29.

In conclusion, high consumption of processed meat was associated with higher overall risk of breast cancer; but this association was driven by post-menopausal breast cancer. After taking account of confounding, red meat consumption was not associated with an overall risk of breast cancer either in UK Biobank or the meta-analysis.

Conflict of interest statement

JPP and NS are members of the UK Biobank steering committee. These facts had no bearing on the study. Otherwise the authors have declared that no competing interests, including financial interests, exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejca.2017.11.022.

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ORIGINAL CONTRIBUTION

Red and processed meat consumption and risk of bladder cancer: a dose–response meta-analysis of epidemiological studies

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Abstract
Background/objectives Several epidemiological studies have analyzed the associations between red and processed meat and bladder cancer risk but the shape and strength of the associations are still unclear. Therefore, we conducted a dose–response meta-analysis to quantify the potential association between red and processed meat and bladder cancer risk.
Methods Relevant studies were identified by searching the PubMed database through January 2016 and reviewing the reference lists of the retrieved articles. Results were combined using random-effects models.
Results Five cohort studies with 3262 cases and 1,038,787 participants and 8 cases–control studies with 7009 cases and 27,240 participants met the inclusion criteria. Red meat was linearly associated with bladder cancer risk in case–control studies, with a pooled RR of 1.51 (95% confidence interval (CI) 1.13, 2.02) for every 100 g increase per day, while no association was observed among cohort studies (*P* heterogeneity across study design = 0.02). Based on both case–control and cohort studies, the pooled relative risk (RR) for every 50 g increase of processed meat per day was 1.20 (95% CI 1.06, 1.37) (*P* heterogeneity across study design = 0.22).
Conclusions This meta-analysis suggests that processed meat may be positively associated with bladder cancer risk. A positive association between red meat and risk of bladder cancer was observed only in case–control studies, while no association was observe in prospective studies.

Keywords Red meat · Processed meat · Bladder cancer · Dose–response · Meta-analysis

Introduction

Bladder cancer is the fifth most common cancer among men and the fourteenth among women with an estimated number of 429,000 cases worldwide in 2012 [1]. Bladder cancer is rather common in developed countries (North America and Europe), and it is more frequent among persons aged 75 or older [2]. Mortality rates have been stable over the last decade with 165,000 estimated deaths in 2012 [1]. A few risk factors have recently been linked to the etiology of bladder cancer. Apart from age and gender, cigarette smoking and specific occupational exposures are considered the most important risk factors [3, 4]. Identification of additional modifiable risk factors such as diet may enhance primary prevention.

Recently two meta-analyses summarized the body of evidence concerning red and processed meat consumption and risk of bladder cancer [5, 6]. Results from the review by Wang et al. [5] indicated an increased risk of bladder cancer of 17 and 10% for high red meat and high processed meat consumption, respectively. The more recent review by

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Li et al. [6], on the other hand, found a significant association for processed meat, with a 22% increased risk of bladder cancer for high consumption but not for red meat consumption. Both meta-analyses, however, were based only on contrasting risk estimates for the highest vs. the lowest category of meat consumption, and this has some limitations when the exposure distribution vary substantially across studies. In the review by Li et al. [6], one of the included studies [7] conducted in Uruguay, for instance, used 0–150 g/day of red meat consumption (median of 85 g/day) as the lowest category. In another study conducted in the USA [8], >58.5 g/day was the highest category for red meat consumption.

Our aim is to describe variation in bladder cancer risk across the whole range of the exposure distribution. A dose–response analysis is more efficient and less sensitive to heterogeneity of the exposure across different study populations. Therefore, we conducted a dose–response meta-analysis to clarify and quantify the potential association between red and processed meat and bladder cancer risk.

Materials and methods

Literature search and selection

Eligible studies were identified by searching the PubMed database through July 2016, with the terms ["bladder" AND ("carcinoma" or "cancer" or "tumor" OR "neoplasms")] AND ("meat" or "beef" or "pork" or "lamb"). In addition, the reference lists of the retrieved articles were examined to identify additional reports. The search was restricted to studies written in English and carried out in human. We performed this meta-analysis accordingly to the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) guidelines [9]. Two authors (A.C. and A.D.) independently retrieved the data from studies on the association between red and processed meat and risk of bladder cancer. Discrepancies were discussed and resolved.

Studies were eligible if they met the following criteria: (1) the study was a cohort or case–control study; (2) the exposure of interest was red meat and/or processed meat; (3) the outcome was incidence of bladder cancer; (4) the authors reported measures of association (hazard ratio, relative risk, odds ratio) with the corresponding confidence intervals for three or more categories for red or processed meat consumption. In case of multiple reports on the same study population, we included only the most comprehensive or recent one.

Data extraction

From each study, we extracted the following information: first author's surname, year of publication, study design, country where the study was conducted, study period, exposure definition, unit of measurement, number of cases, study size, confounding variables, and measure of associations for all the categories of meat consumption together with their confidence intervals. Given the low prevalence of bladder cancer, the odds ratios were assumed approximately the same as the relative risks (RRs). When several risk estimates were available, we included those reflecting the greatest degree of adjustment.

Statistical analysis

We used the method described by Greenland and Longnecker [10] and Orsini et al. [11] to reconstruct study-specific trend from aggregated data, taking into accounts the covariance among the log RR estimates. Risk estimates from studies not reporting information about the number of deaths and study size did not allow reconstruction of the covariance and were assumed independent. Potential nonlinear associations were assessed by use of restricted cubic splines with three knots located at the 10th, 50th, and 90th percentiles of the exposure distribution. An overall P value was calculated by testing that the regression coefficients were simultaneously equal to zero. A P value for nonlinearity was obtained by testing that the coefficient of the second spline term was equal to zero [12].

Since studies used different units to express meat consumption (e.g., servings/day, grams/day, grams per 1000 kcal/day), we converted frequency of consumption using 120 and 50 g as the average portion sizes for red and processed meat, respectively. We chose those values in accordance with previous meta-analyses on meat consumption and other types of cancer [13, 14] and results from both the Continuing Survey of Food Intakes by Individuals [15] and the European Prospective Investigation into Cancer and Nutrition [16]. Meat consumption reported in grams per 1000 kcal/day was converted to g/day using the average energy intake reported in the original articles. Within each exposure category, the median or mean value was assigned to the corresponding RRs. If not reported, we assigned the midpoint of the upper and lower boundaries as average consumption. If the upper bound of the highest category was not reported, we assumed that the category had the same width as the contiguous one. When RRs were reported only for single food items (e.g., separately for beef and pork), we combined them using a fixed-effects model and used the pool estimate as summary measure.

A random-effects meta-analysis was adopted to acknowledge heterogeneity across study findings. Statistical heterogeneity was further assessed by using the Q test (defined as a P value less than 0.10) and quantified by R_b statistic [17]. Meta-regression models were employed to explain residual heterogeneity. Differences in dose–response curves between subgroups of studies were tested

as described by Berlin et al. [18]. Evaluation of goodness-of-fit for the final models was assessed using the set of tools presented by Discacciati et al. [19]. Publication bias was investigated using the Egger asymmetry test [20].

We performed sensitivity analyses (1) excluding studies where red meat definition included also some items of processed meat; (2) excluding studies that did not adjust for energy intake; (3) evaluating alternative average portion sizes for red meat (100 and 140 g) and processed meat (30 and 70 g) consumption. All statistical analyses were conducted with the dosresmeta [21] and metafor [22] packages in R (R Foundation for Statistical Computing, Vienna, Austria) [23]. *P* values less than 0.05 were considered statistically significant.

Results

Literature search

The search strategy identified 146 articles, 108 of which were excluded after review of the title or abstract (Fig. 1). Of the 38 eligible papers 14 were excluded because they did not meet the inclusion criteria (not original articles, outcome different from bladder cancer, or not reporting risk estimates with their confidence intervals). The reference lists of the remaining 24 articles were checked for additional pertinent reports, and 5 additional papers were identified. We further excluded 16 additional articles: 8 presented duplicated publications [24–31]; 3 analyzed bladder and other urinary cancer together [32–34]; 3 did not report enough data for a dose–response analysis [35–37]; and 2 did not report results for red or processed meat consumption [16, 38]. Thus, the meta-analysis included 13 independent epidemiological studies [7, 8, 31, 39–49].

Study characteristics

The main characteristics of the 13 epidemiological studies included in the meta-analysis are presented in Table 1. Five cohort studies [39–43] with 3262 cases and 1038,787 participants and 8 cases–control studies, of which 4 population-based [8, 44, 46, 47] and 4 hospital-based [7, 45, 48, 49], with 7009 cases and 27,240 participants evaluated

Fig. 1 Selection of studies for inclusion in a meta-analysis of red and processed meat consumption and risk of bladder cancer 1966–2016

Table 1 Characteristics of epidemiological studies of meat consumption and risk of bladder cancer in a meta-analysis, 1966–2016

References	Study name	Country	Study period	No. of cases	Study size	Exposure definition	Exposure contrasts	RR (95% CI)	Adjustment variables
Cohort									
Jakszyn [39]	European Prospective Investigation into Cancer and Nutrition	Europe		1001	481,419	Red meat (fresh and processed)	Red meat		Age, gender, center, educational level, BMI (as continuous variable), smoking status, lifetime intensity of smoking (number of cigarettes per day), time since quitting or duration of smoking, and total energy intake
							57.86–91.42 g/day versus 0–57.86 g/day	1.2 (0.96–1.49)	
							91.42–130.63 g/day versus 0–57.86 g/day	1.14 (0.91–1.41)	
							130.63–754.79 g/day versus 0–57.86 g/day	1.15 (0.9–1.45)	
Ferrucci [40]	NIH-AARP Diet and Health Study	USA	1995–2004	854	300,933	Red meat (bacon, beef, cold cuts, ham, hamburger, hot dogs, liver, pork, sausage, and steak) and processed meat (bacon, sausage, luncheon meats, ham, and hot dogs)	Red meat		Age (continuous, years), sex, smoking (never, quit 10 years ago, quit 5–9 years ago, quit 1–4 years ago, quit <1 year ago, or 20 cigarettes/day, 20–40 cigarettes/day, >40 cigarettes/day), and intakes of fruit (continuous, cup equivalents/1000 kcal), vegetables continuous, cup equivalents/1000 kcal), beverages (continuous, mL/day; sum of beer, coffee, juice, liquor, milk, soda, tea and wine), and total energy (continuous, kcal/day)
							20.9 g per 1000 kcal versus 9.5 g per 1000 kcal	0.99 (0.78–1.25)	
							30.7 g per 1000 kcal versus 9.5 g per 1000 kcal	1.05 (0.83–1.33)	
							42.1 g per 1000 kcal versus 9.5 g per 1000 kcal	0.97 (0.77–1.23)	
							61.6 g per 1000 kcal versus 9.5 g per 1000 kcal	1.22 (0.96–1.54)	
							Processed meat		
							4.3 g per 1000 kcal versus 1.6 g per 1000 kcal	1.09 (0.85–1.39)	
							7.4 g per 1000 kcal versus 1.6 g per 1000 kcal	1.1 (0.86–1.41)	
							12.1 g per 1000 kcal versus 1.6 g per 1000 kcal	1.28 (1.01–1.62)	
							22.3 g per 1000 kcal versus 1.6 g per 1000 kcal	1.10 (0.86–1.40)	
Larrson [41]	Swedish Mammography Cohort and the Cohort of Swedish Men	Sweden	1998–2007	485	82,002	Red meat (meatballs or hamburger, beef, pork, veal, kidney, and liver) and processed meat (sausage, ham, salami, and cold cuts)	Red meat		Age, sex, education, smoking status, pack-years of smoking, and total energy intake
							1–4 servings/week versus 0–3 servings/month	1.11 (0.81–1.52)	
							≥5 servings/week versus 0–3 servings/month	1.00 (0.71–1.41)	
							Processed meat		
							1–4 servings/week versus 0–3 servings/month	0.87 (0.68–1.11)	
							≥5 servings/week versus 0–3 servings/month	1.91 (0.80–1.28)	
Michaud [42]	Health Professionals Follow-Up Study and the Nurses' Health Study	USA	1986–2002 and 1976–2002	808	135,893	Red meat (hamburger, beef, pork, lamb as main or mixed dish) and processed meats (bacon, hot dogs, sausage, salami, bologna)	Hamburger		Age, caloric intake (quintiles), and pack-years of smoking and for geographic region

Table 1 continued

References	Study name	Country	Study period	No. of cases	Study size	Exposure definition	Exposure contrasts	RR (95% CI)	Adjustment variables
							0 serving/month versus 1–3 servings/month	0.99 (0.72–1.36)	
							1 serving/week versus 1–3 servings/month	0.86 (0.68–1.08)	
							2–4 servings/week versus 1–3 servings/month	0.91 (0.70–1.17	
							Beef, pork, or lamb (main dish)		
							0 serving/month versus 1–3 servings/month	1.35 (0.94–1.96)	
							1 serving/week versus 1–3 servings/month	1.01 (0.78–1.33)	
							2–4 servings/week versus 1–3 servings/month	1.11 (0.85–1.45)	
							≥5 servings/week versus 1–3 servings/month	0.93 (0.57–1.52)	
							Beef, pork, or lamb (sandwich or mixed dish)		
							0 serving/month versus 1–3 servings/month	1.06 (0.79–1.43)	
							1 serving/week versus 1–3 servings/month	0.83 (0.65–1.06)	
							2–4 servings/week versus 1–3 servings/month	1.26 (0.98–1.63)	
							≥5 servings/week versus 1–3 servings/month	0.95 (0.51–1.75)	
							Hamburger:		
							0 serving/month versus 1–3 servings/month	1.07 (048–2.41)	
							1 serving/week versus 1–3 servings/month	1.13 (0.80–1.60)	
							2–4 serving/week versus 1–3 servings/month	0.96 (0.66–1.38)	
							Beef, pork, or lamb (main dish):		
							0 serving/month versus 1–3 servings/month	2.28 (0.88–5.92)	
							1 serving/week versus 1–3 servings/month	1.35 (0.76–2.39)	
							2–4 servings/week versus 1–3 servings/month	1.23 (0.71–2.11)	
							≥5 servings/week versus 1–3 servings/month	1.01 (0.56–1.65)	
							Beef, pork, or lamb (sandwich or mixed dish)		
							0 serving/month versus 1–3 servings/month	1.61 (0.92–2.81)	
							1 serving/week versus 1–3 servings/month	1.03 (0.75–1.41)	
							2–4 servings/week versus 1–3 servings/month	0.92 (0.66–1.27)	
							≥5 servings/week versus 1–3 servings/month	0.83 (0.40–1.71)	
							Processed meats (e.g., sausage, salami, bologna)		
							1–3 servings/month versus 0 serving/month	0.98 (0.76–1.25)	
							1 serving/week versus 0 serving/month	0.94 (0.71–1.23)	
							2–4 servings/week versus 0 serving/month	0.98 (0.74–1.30)	
							≥5 servings/week versus 0 serving/month	1.09 (0.71–1.69)	
							Bacon		
							1–3 servings/month versus 0 serving/month	1.08 (0.86–1.37)	
							1 serving/week versus 0 serving/month	1.09 (0.84–1.41)	
							2–4 servings/week versus 0 serving/month	1.10 (0.82–1.49)	

Table 1 continued

References	Study name	Country	Study period	No. of cases	Study size	Exposure definition	Exposure contrasts	RR (95% CI)	Adjustment variables
							≥5 servings/week versus 0 serving/month	1.63 (1.02–2.62)	
							Hot dog		
							1–3 servings/month versus 0 serving/month	1.02 (0.83–1.25)	
							1 serving/week versus 0 serving/month	1.02 (0.78–1.34)	
							2–4 servings/week versus 0 serving/month	0.86 (0.58–1.27)	
							Processed meats (e.g., sausage, salami, bologna)		
							1–3 servings/month versus 0 serving/month	1.07 (0.76–1.52)	
							1 serving/week versus 0 serving/month	1.25 (0.86–1.84)	
							2–4 servings/week versus 0 serving/month	0.98 (0.65–1.46)	
							≥5 servings/week versus 0 serving/month	0.81 (0.40–1.63)	
							Bacon		
							1–3 servings/month versus 0 serving/month	0.90 (0.65–1.25)	
							1 serving/week versus 0 serving/month	1.06 (0.74–1.51)	
							2–4 servings/week versus 0 serving/month	1.00 (0.67–1.51)	
							≥5 servings/week versus 0 serving/month	1.48 (0.70–3.16)	
							Hot dog		
							1–3 servings/month versus 0 serving/month	0.91 (0.66–1.24)	
							1 serving/week versus 0 serving/month	0.89 (0.63–1.27)	
							2–4 servings/week versus 0 serving/month	0.77 (0.47–1.24)	
Nagano [43]	Life-Span Study	Japan	1979–1993	114	38,540	Red meat and processed meat (ham, sausage)	Red meat		Age, gender, radiation dose, smoking status, education level, body mass index, and calendar time
							2–4 servings/week versus 0–1 serving/week	0.68 (0.45–1.04)	
							5+ servings/week versus 0–1 serving/week	1.13 (0.53–2.19)	
							Ham and sausage		
							1 serving/week versus 0 serving/week	0.54 (0.31–0.92)	
							2+ servings/week versus 0 serving/week	0.73 (0.42–1.28)	

Table 1 continued

References	Study name	Country	Study period	No. of cases	Study size	Exposure definition	Exposure contrasts	RR (95% CI)	Adjustment variables
Case–control									
Catsburg [44]		USA	1987–1996	1660	3246	Processed meat (fried bacon, ham, salami, pastrami, corned beef, bologna, other lunch meats, hot dogs and polish sausage)	Processed meat		Age, sex, BMI (underweight/normal <25, overweight 25–30, obese >30), race/ethnicity (non-Hispanic white/Hispanic/black or other), education (high school/1- to 4-year college/grad school), history of diabetes (yes/no), total vegetable intake per day, vitamin A intake (IU per day), vitamin C intake (mg per week), carotenoid intake (mcg per day), total servings of food per day, smoking duration (years smoked) and smoking intensity (cigarettes per day)
							1–2 times a week versus < Once a week	0.96 (0.76–1.23)	
							3 times a week versus < Once a week	1.11 (0.87–1.41)	
							4–6 times a week versus < Once a week	1.23 (0.96–1.58)	
							>1 time a day versus < Once a week	0.97 (0.74–1.27)	
Isa [45]		China	2005–2008	487	956	Red meat and preserved meat	2–4 times/week versus ≤1 times/week	1.20 (0.90–2.10)	Sex, age (categorical), smoking status (categorical), smoking duration (continuous), smoking amount (continuous), and other food groups
							≥5 times/week versus ≤1 times/week	1.80 (1.10–3.00)	
							Preserved meat		
							<1 times/month versus never	1.60 (1.00–2.80)	
							1–3 times/month versus never	1.70 (0.90–3.10)	
							1 times/week versus never	2.20 (1.00, 4.7)	
Wu [46]		USA	2001–2004 and 2002–2004	1171	2535	Red meat (beef, veal, pork, and lamb) and processed meat (ham, bacon, sausage, hot dog, cold cuts, turkey sausages and hot dogs, and poultry cold cuts)	Red meat		Gender, age (0–54, 55–64, 65–74, 75+), region, race (White/other), Hispanic status, smoking status (never, occasional, former, current), usual BMI (continuous), and total energy (kcal per day—continuous)
							27.6 g per 1000 kcal versus 17.2 per 1000 kcal	0.97 (0.76–1.24)	
							37.4 g per 1000 kcal versus 17.2 per 1000 kcal	1.04 (0.81–1.33)	
							53 g per 1000 kcal versus 17.2 per 1000 kcal	1.14 (0.89–1.46)	
							Processed meat		
							6.1 g per 1000 kcal versus 2.9 per 1000 kcal	1.01 (0.78–1.30)	
							10.1 g per 1000 kcal versus 2.9 per 1000 kcal	1.19 (0.92–1.53)	
							18.4 g per 1000 kcal versus 2.9 per 1000 kcal	1.28 (1.00–1.65)	
Lin [8]		USA	1999	884	1762	Red meat (beef, veal, lamb, pork and game) and processed meat (hot dogs or franks, sausage or chorizo)	Red meat		Age, sex, ethnicity, smoking status, pack-year of smoking, energy intake, total vegetable intake, total fruit intake, and BMI
							0.55–1.10 once versus <0.55 once	1.17 (0.87–1.58)	
							1.11–2.05 once versus <0.55 once	1.47 (1.09–1.99)	
							≥2.06 once versus <0.55 once	1.95 (1.41–2.68)	
							Processed meat:		
							0.11–0.28 once versus <0.11 once	0.88 (0.66–1.18)	
							0.29–0.61 once versus <0.11 once	0.98 (0.73–1.31)	
							≥0.62 once versus <0.11 once	1.03 (0.76–1.39)	

Table 1 continued

References	Study name	Country	Study period	No. of cases	Study size	Exposure definition	Exposure contrasts	RR (95% CI)	Adjustment variables
Aune [7]		Uruguay	1996–2004	255	2287	Red meat (fresh meat including beef and lamb) and processed meat (hot dogs, sausages, ham, salami, saucisson, mortadella, bacon and salted meat)	10–40 g/day versus 0–10 g/day	1.01 (0.70–1.46)	Age, sex, residence, education, income, interviewer, smoking status, cigarettes per day, duration of smoking, age at starting, years since quitting, alcohol, dairy foods, grains, fatty foods (butter, eggs, custard, cake), fruits and vegetables, fish, poultry, mate drinking, BMI, and energy intake
							>40–258.8 versus 0–10 g/day	1.43 (0.93–2.20)	
Hu [47]		Canada	1994–1997	1209	6248	Red meat (beef, pork, lamb as a main or mixed dish and hamburger) and processed meat (hot dogs, smoked meat, corned beef, bacon and sausage)	Red meat		Age group (20–49, 50–59, 60–69, 70–76), province, education, body mass index (<25, 25–29.9, ≥30), sex, alcohol use (g/day), pack-year smoking, total of vegetable and fruit intake, and total energy intake
							2.1–3.94 times/week versus ≤2 times/week	1.20 (1.00–1.60)	
							3.95–5 times/week versus ≤2 times/week	1.20 (090–1.50)	
							≥5.42 times/week versus ≤2 times/week	1.30 (1.0–1.70)	
							Processed meat:		
							0.95–2.41 times/week versus ≤0.94 times/week	1.20 (1.10–1.60)	
							2.42–5.41 times/week versus ≤0.94 times/week	1.50 (1.10–1.90)	
							≥5.42 times/week versus ≤0.94 times/week	1.60 (1.20–2.10)	
Closas [48]		Spain	1998–2001	912	1785	Red meat (beef, veal, lamb, pork) and processed meat	Red meat:		Age (<55, 55–64, 65–69, 70–74, >74 years old), gender, region, smoking status (never, occasional, former, current), duration of smoking (<20, 20–<30, 30–<40, 40–<50, ≥ 50 years) and quintiles of fruit and vegetable intake
							(20–32) g per 1000 kcal versus <20 g per kcal	1.10 (0.80–1.50)	
							(33–43) g per 1000 kcal versus <20 g per kcal	1.10 (0.80–1.50)	
							(44–58) g per 1000 kcal versus <20 g per kcal	1.00 (0.70–1.30)	
							(>58) g per 1000 kcal versus <20 g per kcal	0.80 (0.60–1.10)	
							Processed meat:		
							(4–9) g per 1000 kcal versus <4 g per kcal	1.40 (1.00–1.90)	
							(10–12) g per 1000 kcal versus <4 g per kcal	1.20 (0.90–1.70)	
							(13–18) g per 1000 kcal versus <4 g per kcal	1.20 (0.80–1.60)	
							(>18) g per 1000 kcal versus <4 g per kcal	1.20 (0.90–1.70)	
Tavani [49]		Italy	1983–1996	431	8421	Red meat (beef, veal and pork)	Red meat		Age, year of recruitment, sex, education, smoking habits and alcohol, fat, fruit and vegetable intakes
							3–6 times/week versus ≤3/week	1.40 (1.20–1.80)	
							≥6 times/week versus ≤3 times/week	1.60 (1.20–2.10)	

the relation between red and/or processed meat and risk of bladder cancer. Two articles [39, 49] reported results only for red meat, while one [44] only for processed meat. Definition of meat and red meat varied across studies but generally included beef, veal, pork, and lamb for red meat, and bacon, ham, salami, sausages, and hot dogs for processed meat. Two cohort studies [39, 40] included also processed meat in the definition of red meat, and one study [42] included only results for specific food items. One study [44] reported results only for liver intake and was not included in the analysis of red meat. Another study [45] analyzed preserved meat consumption and, given the limited range of exposure (up to 1/week), was excluded from the analysis of processed meat.

Only 3 studies [40, 46, 48] considered different cooking methods and doneness levels for meat consumption. None of them found evidence of an association between preparation methods and bladder cancer. Different units were used to report meat consumption: servings/week (7 studies), grams per 1000 kcal per day (3 studies), and grams per day (3 studies). Five studies were conducted in the USA, 4 in Europe, and 1 each in Canada, Uruguay, China, and Japan. All the studies were carried out in both men and women, and only one study [42] reported results separately by gender. All the studies provided measure of associations adjusted for age, gender, and smoking. Four studies did not adjust for energy intake [43–45, 49]. Other common adjusting variables were other food groups (8 studies), BMI (6 studies), education (6 studies). Additional covariates were less consistent across studies.

Association between red meat consumption and risk of bladder cancer

We found a statistically significant association between red meat consumption and risk of bladder cancer ($P = 0.009$; P nonlinearity $= 0.74$) (Online Resource 1). The summary RR for an increment of 100 g per day of red meat was 1.22 (95% CI 1.05, 1.41). There was substantial between-studies heterogeneity ($R_b = 67\%$, $P < 0.01$). Egger's regression test did not suggest the presence of substantial publication bias ($P = 0.14$).

There was statistical heterogeneity according to study design (P for heterogeneity $= 0.02$). The pooled RR restricted to the cohort studies was 1.01 (95% CI 0.97, 1.06) for an increment of 100 g per day of red meat with no significant heterogeneity ($R_b = 0\%$, $P = 0.62$) (Figure 2). The deviance test did not detect lack of fit ($D = 24$, $df = 18$, $P = 0.17$). In case–control studies, the corresponding pooled RR was 1.51 (95% CI 1.13, 2.02) with substantial heterogeneity among studies ($R_b = 81\%$, $P < 0.01$) and overall indication of poor fit ($D = 44$, $df = 18$, $P < 0.01$).

No differences were found according to study location (P for heterogeneity $= 0.7$), units of measurement (P for heterogeneity $= 0.38$), and selection of controls (P for heterogeneity $= 0.65$). Excluding those studies with also processed meat in the definition of red meat, the pooled RRs were 1.27 (95% CI 1.03, 1.57) overall and 0.95 (95% CI 0.82, 1.11) restricted to cohort studies. The pooled RR for an increment of 100 g of red meat per day was 1.14 (95% CI 0.99, 1.31) based on studies that adjusted for energy intake. In the sensitivity analysis for alternative average portion sizes of red meat, the results did not substantially change. The pooled RR for an increment of 100 g of red meat per day was 1.27 and 1.19 for assigned portions of 140 g per day and 100 g per day, respectively.

For an increment of four servings per week of red meat (120 g per servings), the summary RR of bladder cancer was 1.15 (95% CI 1.03, 1.27) overall, 1.01 (95% CI 0.98, 1.04) for cohort studies, and 1.32 (95% CI 1.08, 1.62) for case–control studies.

Association between processed meat consumption and risk of bladder cancer

We found a statistically significant association between processed meat intake and bladder cancer with no departure from linearity ($P = 0.005$, P nonlinearity $= 0.92$) (Online Resource 2). Every 50 g increase in processed meat per week was associated with a 20% (95% CI 6, 37) increase in risk of bladder cancer with moderate heterogeneity ($R_b = 38\%$, $P = 0.07$). Egger's regression test did not detect publication bias ($P = 0.21$). No evidence of lack of fit was observed ($D = 43$, $df = 34$, $P = 0.14$). The test did not detect significant differences between case–control and cohort studies (P for heterogeneity $= 0.22$). Stratified analysis provided a RR of 1.10 (95% CI 0.95, 1.27) and 1.31 (95% CI 1.06, 1.63) for cohort and case–control studies, respectively (Fig. 3).

The associations were similar across strata of study location (P for heterogeneity $= 0.68$), units of measurement (P for heterogeneity $= 0.71$), and selection of controls (P for heterogeneity $= 0.46$). Exclusion of studies that did not adjust for energy intake provided a pooled RR of 1.24 (95% CI 1.07, 1.43). Similar results were observed for alternative average portion sizes of 30 g per day and 70 g per day with pooled RR, respectively, of 1.14 and 1.36 for an increment of 50 g per day of processed meat.

For an increment of four servings per week of processed meat (50 g per servings), the summary RR of bladder cancer was 1.11 (95% CI 1.03, 1.20) overall, 1.06 (95% CI 0.97, 1.15) for cohort studies, and 1.17 (95% CI 1.03, 1.32) for case–control studies.

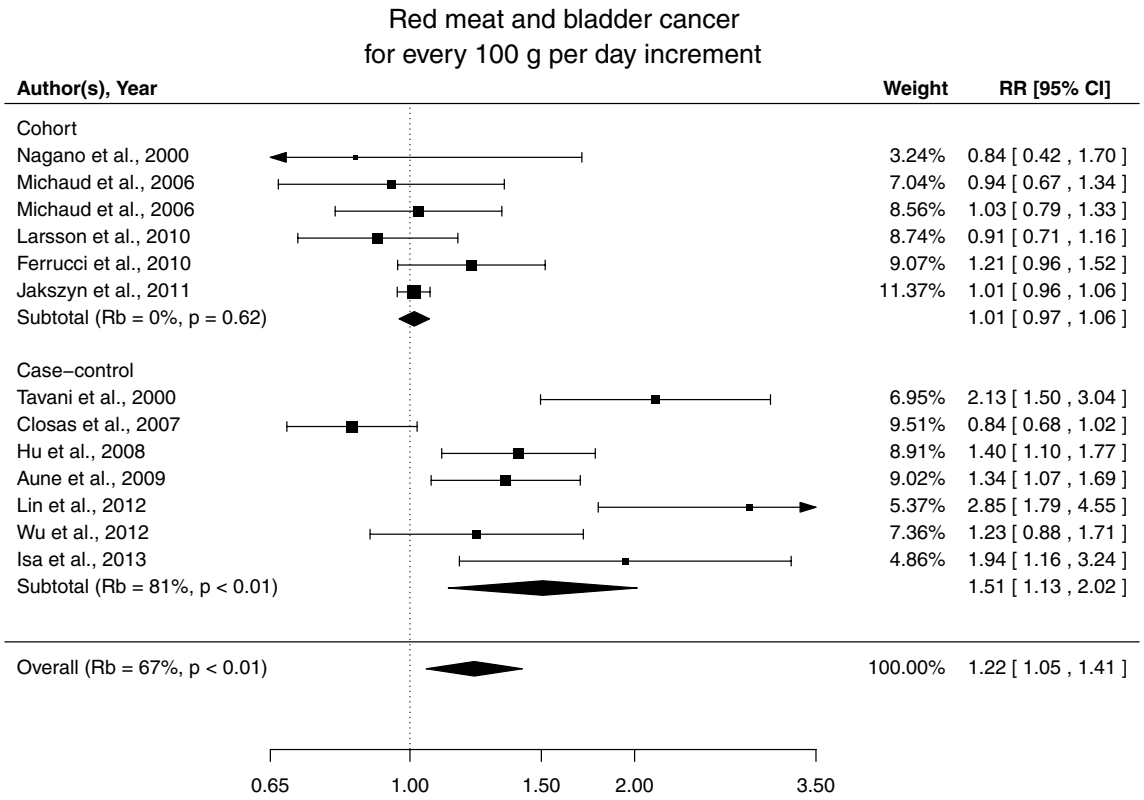

Fig. 2 Relative risks of bladder cancer with 100 g per day increment in red meat consumption separately for cohort and case–control studies

Discussion

Findings from this dose–response meta-analysis of five cohort and eight case–control studies suggest that processed meat consumption is positively associated with risk of bladder cancer. An increment of 50 g of processed meat per day was associated with 20% increased risk of bladder cancer. Red meat consumption was associated with bladder cancer only in case–control studies, with a 51% increased risk of an increment of 100 g per day, while no association was observed among the prospective studies.

Meat, in particular processed meat, is a potential risk factor for several cancers, with the most convincing evidence for colorectal cancer [50]. In 2015, the International Agency for Research on Cancer classified processed meats as carcinogenic to humans (Group 1) and red meat as probably carcinogenic to humans [51]. The contribution of meat to the etiology of bladder cancer may be explained by different mechanisms, given that many nutrients are excreted through the urinary tract [52]. The most established mechanism involves the formation of endogenous nitrosamines from nitrites that are particularly abundant in processed meats [53]. Experimental studies have shown that some nitrosamine metabolites induce bladder tumors in rodents [54, 55]. Further support for at potential role of nitrosamines in bladder carcinogenesis is that cigarette smoking is a strong risk factor for bladder cancer and tobacco smoke is a main source of exogenous exposure to nitrosamines. Consumption of red meat could potentially increase the risk of bladder cancer through heterocyclic amines and polycyclic aromatic hydrocarbons, which can be generated from high temperature cooking [56]. Heterocyclic amines and polycyclic aromatic hydrocarbons have been consistently shown to be carcinogenic in animal studies [56, 57].

A direct comparison with the results of previous reviews [5, 6] is difficult since they were based on study-specific risk estimates for high versus low categories of meat consumption, which varied substantially across studies. The directions of the associations, however, were consistent, even though an association was found only for processed meat in the meta-analysis by Lin et al. [6]. As in the review by Wang et al. [5], case–control studies provided stronger risk estimates as compared to prospective studies.

Among several potential explanations, case–control studies generally assess the exposure after diagnosis, and therefore, recall bias may lead to differential misclassification between cases and controls. Considering the limited knowledge of the role of meat consumption on the development of bladder cancer [44], such classification errors are

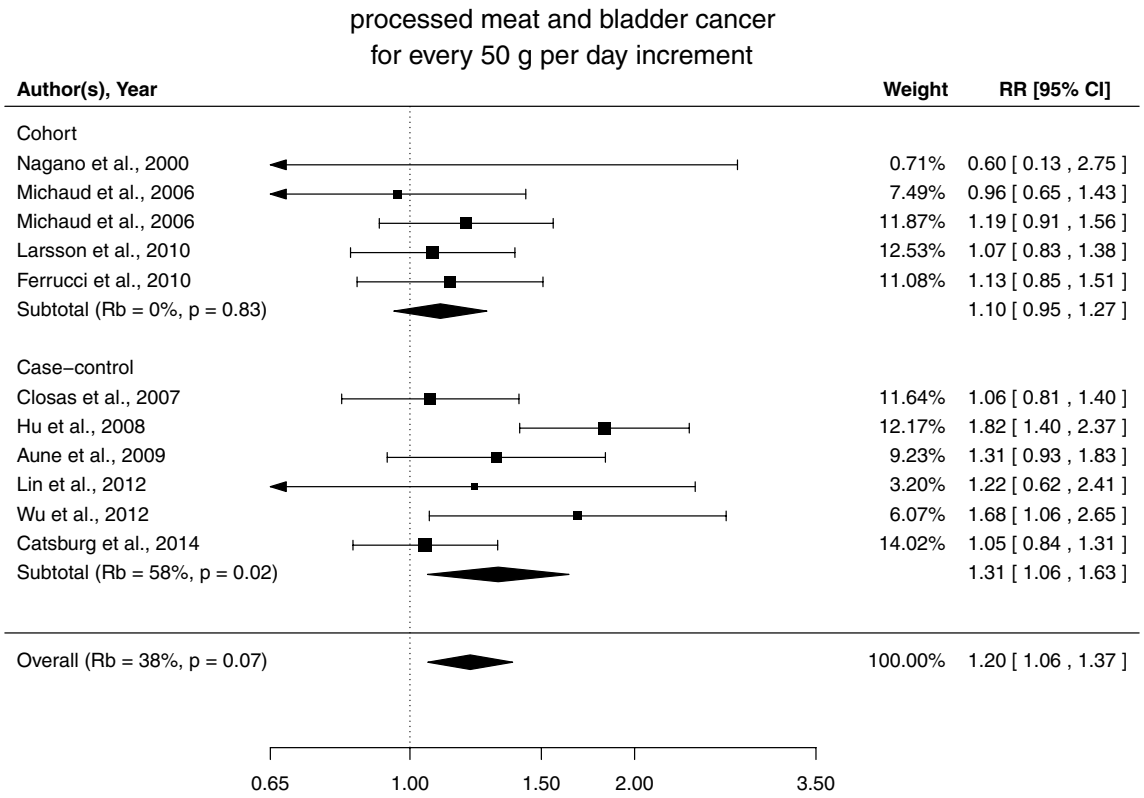

Fig. 3 Relative risks of bladder cancer with 50 g per day increment in processed meat consumption separately for cohort and case–control studies

likely to be similar among cases and controls. On the other hand, half of the control studies used hospital-based controls which may inflate the pooled association in case controls have been recruited for conditions linked with changes in meat consumption. Although based on limited number of studies, we did not observed differences in results between hospital-based and population-based case–control studies. Different participation rates related to exposure or severity of diseases may also be a selection bias among case–control studies. In addition, the time between diagnosis and the exposure assessment is generally shorter for case–control studies; hence, it may not reflect long-term exposure because of changes in dietary patterns. On the other hand, in cohort studies participants may alter their dietary intake during the follow-up, which may bias results toward the null hypothesis of no association. One of the included cohort studies [42] analyzed repeated dietary measurements over time and observed stronger associations when using cumulative update date and when removing participant who had change their meat consumption.

Strength of this review is the dose–response analysis, which better takes into account the quantitative nature and heterogeneity of the exposure. In our analysis, all the information about meat consumption, including intermediate categories, contributed to the pooled associations. Another strength is the large number of cases that provided high statistical power to detect associations of moderate magnitude. Lastly, no evidence of publication bias was observed.

This meta-analysis also had potential limitations. Pooling results from epidemiological studies do not solve the problem of residual confounding, which inherently affects individual studies. All of the included studies, however, adjusted for main known risk factors for bladder cancer such as age, gender, and smoking, and some studies also adjusted for energy intake, BMI, education, and other food groups. Excluding those studies not adjusting for energy intake did not change the overall results, suggesting that energy intake may have a limited impact on developing bladder cancer. Second, red and processed meat definition varied across study and this may partially contribute to the observed heterogeneity. Different units of measurements were also used to report risk estimates for meat consumption, and we had to assume average portion sizes when meat consumption was reported as servings. Nevertheless, stratified analysis for different types of measurements and sensitivity analysis for alternative portion sizes did not find substantial differences in results. Third, it was not possible to investigate the association between different meat-cooking methods and bladder

cancer because only three articles reported such information. However, none of them found an increase in bladder cancer risk with any of the cooking methods. Fourth, statistical heterogeneity was observed in our analysis as in the previous two reviews [5, 6] but was mainly explained by different study design. After stratification, moderate heterogeneity was still observed among case–control studies, while cohort studies provided more homogenous results.

In conclusion, results from this dose–response meta-analysis suggest that processed meat consumption may be positively associated with risk of bladder cancer. Positive association between red meat and risk of bladder cancer was observed only in case–control studies, while no association was observed in prospective studies.

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Authors' contribution All authors (AC, SL, AD, AW, and NO) participated both in the study design and in writing the manuscript. AC and AD participated in the data collection. AC analyzed the data and wrote the manuscript under the supervision of NO. SL and AW interpreted the results and critically reviewed the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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Plasma branched chain/aromatic amino acids, enriched Mediterranean diet and risk of type 2 diabetes: case-cohort study within the PREDIMED Trial

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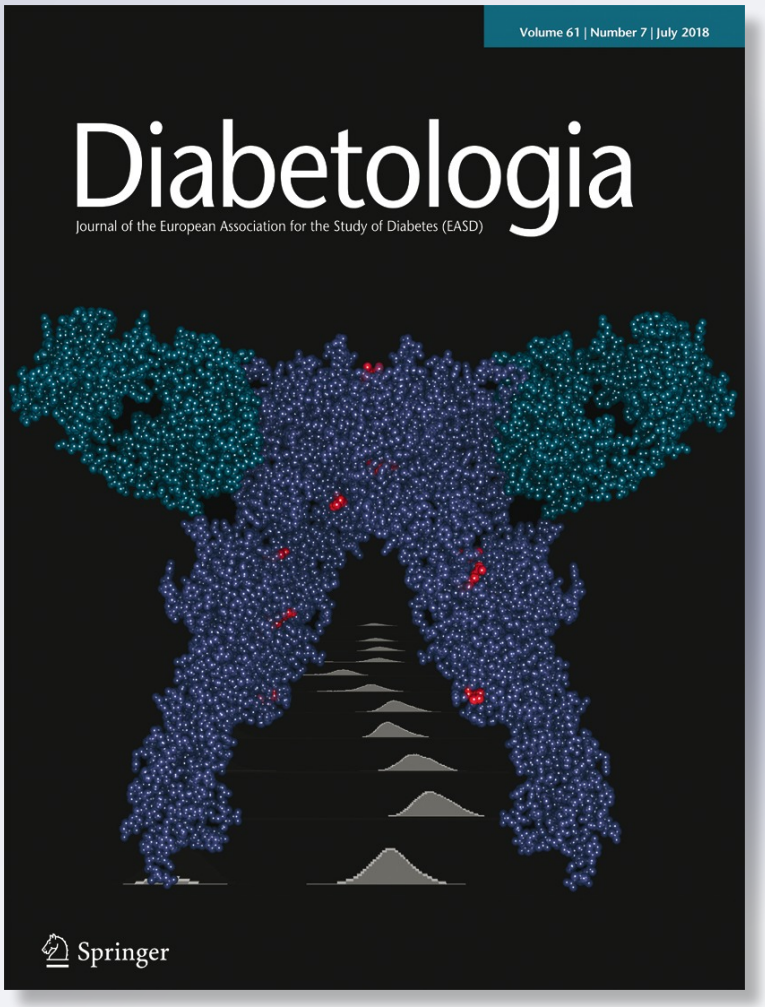

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ARTICLE

Plasma branched chain/aromatic amino acids, enriched Mediterranean diet and risk of type 2 diabetes: case-cohort study within the PREDIMED Trial

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Abstract

Aims/hypothesis Branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) are associated with type 2 diabetes. However, repeated measurements of BCAA/AAA and their interactions with dietary interventions have not been evaluated. We investigated the associations between baseline and changes at 1 year in BCAA/AAA with type 2 diabetes in the context of a Mediterranean diet (MedDiet) trial.

Methods We included 251 participants with incident type 2 diabetes and a random sample of 694 participants (641 participants without type 2 diabetes and 53 overlapping cases) in a case-cohort study nested within the PREvención con DIeta MEDiterránea (PREDIMED) trial. Participants were randomised to a MedDiet+extra-virgin olive oil ($n = 273$), a MedDiet+nuts ($n = 324$) or a control diet ($n = 295$). We used LC-MS/MS to measure plasma levels of amino acids. Type 2 diabetes was a pre-specified secondary outcome of the PREDIMED trial.

Results Elevated plasma levels of individual BCAAs/AAAs were associated with higher type 2 diabetes risk after a median follow-up of 3.8 years: multivariable HR for the highest vs lowest quartile ranged from 1.32 for phenylalanine ([95% CI 0.90, 1.92], p for trend = 0.015) to 3.29 for leucine ([95% CI 2.03, 5.34], p for trend<0.001). Increases in BCAA score at 1 year were associated with higher type 2 diabetes risk in the control group with HR per SD = 1.61 (95% CI 1.02, 2.54), but not in the MedDiet groups (p for interaction <0.001). The MedDiet+extra-virgin olive oil significantly reduced BCAA levels after 1 year of intervention ($p = 0.005$ vs the control group).

Conclusions/interpretation Our results support that higher baseline BCAAs and their increases at 1 year were associated with higher type 2 diabetes risk. A Mediterranean diet rich in extra-virgin olive oil significantly reduced the levels of BCAA and attenuated the positive association between plasma BCAA levels and type 2 diabetes incidence.

Clinical trial number: SRCTN35739639 (www.controlled-trials.com)

Keywords Aromatic amino acids · Branched-chain amino acids · Mediterranean diet · Type 2 diabetes

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00125-018-4611-5) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

AAA	Aromatic amino acid
BCAA	Branched-chain amino acid
EVOO	Extra-virgin olive oil
MedDiet	Mediterranean diet (trial intervention)
MET	Metabolic equivalent task
mTOR	Mammalian target of rapamycin
PREDIMED	PREvención con DIeta MEDiterránea

Research in context

What is already known about this subject?

- Elevated levels of plasma branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) are associated with insulin resistance, impaired glucose tolerance and type 2 diabetes

What is the key question?

- Does a 1 year enriched Mediterranean diet reduce plasma BCAA and AAA levels? Is this reduction linked to reduced risk of type 2 diabetes?

What are the new findings?

- Higher baseline BCAAs and their increases after 1 year were associated with a higher risk of type 2 diabetes
- A Mediterranean diet enriched with extra-virgin olive oil was associated with lower risk of diabetes in participants with low baseline levels of BCAA and AAA and this intervention also reduced circulating levels of BCAA after 1 year

How might this impact on clinical practice in the foreseeable future?

- Our results shed light on a potential role of BCAAs/AAAs in the development of type 2 diabetes and the benefits of a Mediterranean diet to modulate their adverse effects

Introduction

Leucine, isoleucine and valine are branched-chain amino acids (BCAAs) that are derived from the diet and vital for normal growth and function at the cell and organism levels [1]. High-throughput techniques for metabolomic profiling have identified BCAAs as potential biomarkers for type 2 diabetes risk [2]. Elevated levels of plasma BCAAs have been associated with obesity, insulin resistance, impaired glucose tolerance and type 2 diabetes [3, 4]. Similarly, baseline phenylalanine and tyrosine are aromatic amino acids (AAAs) associated with higher risk of incident type 2 diabetes [5].

In a meta-analysis [6], we reported positive associations between elevated plasma or serum levels of BCAA and AAA with higher type 2 diabetes risk. The pooled RR per SD of each amino acid ranged from 1.26 (95% CI 1.10, 1.44) to 1.36 (95% CI 1.24, 1.48) [6]. However, none of these studies or subsequent studies [7–13] used repeated measurements of these amino acids over time nor evaluated how dietary interventions can influence changes in the levels of these plasma amino acids and risk of type 2 diabetes. This more dynamic assessment is important because a decreased uptake and an increased release of amino acids from skeletal muscle can also be a consequence of increased protein catabolism with underlying insulin resistance [14]. Alternatively, circulating amino acids may disrupt signalling in the liver and skeletal muscle and may directly promote insulin resistance or promote the destruction of pancreatic beta cells and eventually lead to the onset of type 2 diabetes [4].

In this study we tested the following four hypotheses in a case-cohort study of participants, without type 2 diabetes at baseline, nested within the PREvención con DIeta Mediterránea (PREDIMED) trial: (1) baseline plasma levels of BCAA and AAA are positively associated with higher type 2 diabetes risk; (2) increases in these amino acids at 1 year are associated with a higher subsequent risk of type 2 diabetes; (3) a Mediterranean-style diet (MedDiet) can attenuate the positive association between BCAAs/AAAs and type 2 diabetes; and (4) a MedDiet intervention of one year duration is able to reduce the plasma levels of these amino acids.

Methods

Our study was nested, as an unstratified case-cohort study, within the PREDIMED study (www.predimed.es), a Spanish primary cardiovascular disease prevention trial using a Mediterranean diet as the main intervention. The methods and design of PREDIMED were previously reported in detail elsewhere [15]. Briefly, 7447 participants (men aged 55 to 80 years and women aged 60 to 80 years) were randomly allocated to three equally sized groups: (1) a MedDiet supplemented with extra-virgin olive oil (EVOO); (2) a MedDiet supplemented with mixed nuts; or (3) a control diet where participants were advised to reduce the intake of all types of fat. The recruitment took place across 11 recruiting centres between 2003 and 2009 and the study was stopped early in July 2011 when a preplanned interim analysis provided early evidence of significant benefits for the two MedDiets.

Participants were selected for the PREDIMED trial because they had either type 2 diabetes or had three or more major cardiovascular risk factors. In the full PREDIMED cohort, 3541 participants did not have type 2 diabetes at baseline. Among them, we observed 273 incident cases of type 2 diabetes, a pre-specified secondary outcome of the PREDIMED trial. Participants who were randomised to the MedDiet+

EVOO (or both MedDiets combined) had a significantly lower risk of type 2 diabetes compared with the control group [16].

In the present study we performed additional metabolomic measurements in a subpopulation of the PREDIMED trial. Specifically, this case-cohort study comprises a random selection of 694 participants without diabetes (approximately 20%) from the eligible volunteers of the PREDIMED cohort who were free of diabetes at baseline and had available plasma samples, together with all incident cases of type 2 diabetes that occurred during a median of 3.8 years of intervention (samples were unavailable for 22 out of the 273 participants with incident type 2 diabetes occurring in the PREDIMED trial; Fig. 1). Of the 892 participants included in our analyses, 251 were incident cases of type 2 diabetes and 641 (plus 53 overlapping participants) were selected from the random 20% subcohort. In addition, 663 participants (505 without diabetes and 158 cases that occurred after 1 year of follow-up) had follow-up samples at 1 year and were included in the '1 year increases' analyses. The Research Ethics Committees for each of the recruitment centres approved the study protocol and all participants provided written informed consent.

Covariate assessment At baseline and at yearly follow-up visits, a questionnaire about lifestyle variables, educational achievement, personal history of illnesses, medication use and family history of disease was administered. Physical activity was assessed using the validated Spanish version of the 'Minnesota Leisure-Time Physical Activity' questionnaire [17]. Participants were considered to have hypercholesterolemia or hypertension if they had previously been diagnosed and/or they were being treated with cholesterol-lowering or antihypertensive agents, respectively. Trained personnel ascertained anthropometric and blood pressure measurements.

Study samples and metabolite profiling Fasting blood samples were collected at baseline and yearly thereafter during

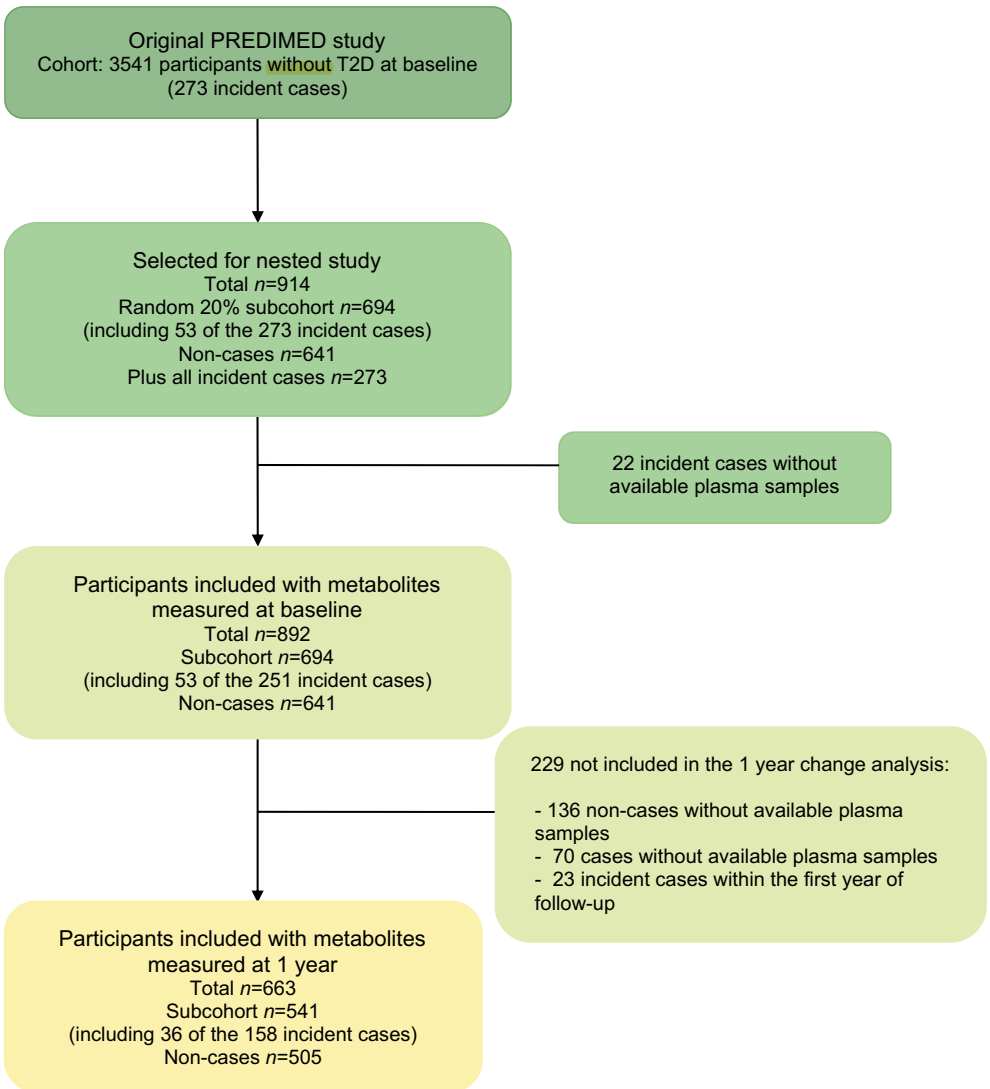

Fig. 1 Flow chart showing the case-cohort design. T2D, type 2 diabetes

follow-up. After an overnight fast, plasma EDTA tubes were collected and aliquots were coded and kept refrigerated until they were stored at −80°C. In June 2015, pairs of samples (baseline and first year visits from each participant) were randomly ordered and shipped on dry ice to the Broad Institute (Boston, MA, USA) for the metabolomic analyses. Amino acids, acylcarnitines and other polar plasma metabolites were profiled using liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [18–20]. For further details, please refer to the electronic supplementary material (ESM) Methods.

Additionally, fasting glucose and insulin were determined in plasma samples, both at baseline and 1 year. Glucose was measured using an enzymatic method to convert glucose to 6-phosphogluconate (ADVIA Chemistry Systems, Tarrytown, NY, USA). The intra- and inter-assay coefficients of variation were 1.2 and 1.6. Insulin concentrations were measured using an immunoenzymometric assay (ADVIA Chemistry Systems) with and intra- and inter-assay coefficient of variation equal to 3.7 and 4.4, respectively. Insulin resistance was calculated using HOMA-IR (insulin resistance = fasting insulin × fasting glucose/155.25, where insulin is in pmol/l and glucose is in mmol/l).

Clinical assessment The PREDIMED protocol included type 2 diabetes as a pre-specified secondary endpoint of the trial among participants initially free of diabetes. The adjudication for new diagnoses of incident cases of type 2 diabetes during follow-up was made in a blinded assessment conducted by the Clinical Endpoint and Adjudication of Events Committee of PREDIMED; an ad hoc panel of medical doctors, and is described elsewhere [15, 16]. The criteria of the American Diabetes Association [21], namely two confirmations of fasting plasma glucose ≥7.0 mmol/l or 2 h plasma glucose ≥11.1 mmol/l after a 75-g oral glucose load, were used to adjudicate confirmed cases. Only confirmed cases were included in the statistical analyses.

Statistical analysis Individual BCAA values were normalised and scaled to multiples of 1 SD using the rank-based inverse normal transformation [22]. We fitted weighted Cox regression models using Barlow weights to account for the over-representation of participants with type 2 diabetes, as recommended for case-cohort designs [23]. We calculated HR and their 95% CIs for type 2 diabetes by quartiles of the amino acids and also for each SD as a continuous variable. Follow-up time was calculated from the date of enrolment to either the date of diagnosis of type 2 diabetes or to the date of the last visit or the end of the follow-up period for participants without type 2 diabetes (1 December 2010). We fitted crude models adjusting for age (years), sex, intervention group and multivariable models. All models were stratified by recruitment centre. Multivariable-adjusted models were additionally adjusted for smoking status (never/current/former), body mass index (BMI, kg/m^2), leisure-time physical activity (metabolic equivalent task [MET]-min/day), hypertension and dyslipidaemia. In a secondary analysis, we additionally adjusted for plasma glucose (adding a quadratic term to account for the departure from linearity) because blood glucose was likely to be not only a confounder but also an intermediate link in the causal pathway between BCAAs or AAAs and risk of type 2 diabetes. As an ancillary analysis, we additionally adjusted for an acylcarnitine score calculated as the sum of raw values of all these metabolites and categorised as quartiles. We used a simple imputation method (using age, sex, BMI and waist circumference as predictors) to estimate baseline glucose in 15 participants with missing values from glucose.

We calculated a baseline BCAA score as the sum of leucine, isoleucine and valine, and baseline AAA score as the sum of phenylalanine and tyrosine. We used the simple sum of normalised values of these metabolites.

Quartile cut-off points for amino acids and their scores were generated based on the distributions of BCAAs among participants without diabetes. We conducted tests of linear trend by examining an ordinal score based on the median value in each quartile of BCAAs in the multivariable models.

We conducted joint analyses and interactions tests for the BCAA or AAA score and the intervention groups (MedDiet+EVOO and MedDiet+nuts vs control group) both with baseline levels. We considered as the reference group those participants who were randomised to the MedDiet+EVOO and with low BCAA or AAA scores (<percentile 50). The likelihood ratio test was used to assess the significance of interaction between the intervention and the BCAA or AAA score.

We also examined how changes in the individual amino acid levels at 1 year and the overall BCAA and AAA scores were associated with diabetes risk. We used only cases of type 2 diabetes occurring after 1 year follow-up as an outcome in a multivariable-adjusted Cox regression model. With respect to individual metabolites, we first calculated the difference between baseline and levels at 1 year and then normalised this difference using the inverse normal transformation. For changes in the scores at 1 year, we summed changes in the three metabolites at 1 year and subsequently normalised their sum. We additionally categorised the change in amino acids at 1 year into three groups: decrease, no change or increase. The 'no change' category included changes lower than 1 SD, a 'decrease' was considered as a reduction greater than 1 SD and an 'increase' was defined as an elevation greater than 1 SD. We repeated the multivariable-adjusted Cox models using these three categories as the main exposure.

We evaluated the association between the intervention group and changes in individual metabolites at 1 year or in the overall BCAA/AAA scores using a multivariable-adjusted ANOVA model. In this model, we adjusted for age (years), sex (male/female), BMI (kg/m^2), smoking (never/current/

former), leisure-time physical activity (MET-min/day), dyslipidaemia, hypertension and baseline fasting glucose.

We also assessed whether the association between the intervention group and changes in these metabolites at 1 year or the overall scores were mediated by changes in insulin or HOMA-IR. To assess this potential mediating effect, we first performed a multivariable linear regression to test the association between the intervention group and changes in insulin or HOMA-IR at 1 year (using both insulin and HOMA-IR as dependent variables). Second, we assessed the association between the intervention group and changes in metabolites at 1 year after additionally adjusting for insulin or HOMA-IR. We excluded from these analyses participants with a diagnosis of type 2 diabetes during the first year of follow-up.

For the analyses assessing changes in HOMA-IR or in insulin at 1 year, we used both complete case analyses and multiple imputation methods to replace the values of insulin or HOMA-IR in participants with missing data for these variables ($n = 160$). We used the multivariable normal method with the command 'mi impute' in Stata version 13.1 (Stata Corp., College Station, TX, USA) and we ran 20 sets of random imputations. This method uses multivariate data augmentation to impute missing values of continuous variables. Predictors for imputing the missing values of insulin and HOMA-IR were age, sex, BMI, waist circumference, baseline glucose levels, incident diabetes status, group of intervention and changes in leucine, isoleucine, valine, phenylalanine and tyrosine at 1 year, as recommended by methodologists [24].

Finally, we examined the association between changes in HOMA-IR at 1 year with BCAA and AAA scores (quartiles) adjusting for age, sex, intervention group, smoking status, BMI (kg/m^2), leisure-time physical activity (MET-min/day), hypertension, dyslipidaemia and baseline plasma glucose. In a second model we additionally adjusted for changes in BMI at 1 year. In these analyses, we excluded participants with type 2 diabetes diagnosed during the first year of follow-up.

All statistical analyses were performed using Stata version 13.1 (Stata Corp.).

Results

Participant characteristics Table 1 presents the characteristics of participants included in our analyses according to whether they developed type 2 diabetes during follow-up and according to extreme quartiles of the amino acid scores. Cases of type 2 diabetes were more likely to be current smokers, to have hypertension and higher average BMI, waist circumference and higher baseline blood glucose levels. The proportion of women was also lower in cases than in participants without diabetes.

Participants in the top quartile of the BCAA score (vs the lowest quartile) were more likely to be men and current smokers. They also exhibited higher than average values of BMI, waist circumference and blood glucose. On the other hand, they were more physically active and younger. Differences between extreme quartiles of the AAA score were smaller, showing only significantly higher BMI and waist circumference.

Baseline associations between BCAAs/AAAs and type 2 diabetes Table 2 presents the associations between the baseline BCAA and AAA scores with the incidence of type 2 diabetes. The positive associations between each of the two baseline scores (BCAA and AAA) and the risk of incident type 2 diabetes were statistically significant in the total sample and also in the control and MedDiet+EVOO groups. The positive association between baseline plasma levels of BCAA and type 2 diabetes was considerably attenuated in the MedDiet+nuts groups. When we considered a 2 degree of freedom interaction (Table 2) or when we restricted our analyses to the comparison between the MedDiet+EVOO vs the control group, the interactions were statistically significant. The interaction between the intervention with MedDiet+EVOO and the baseline BCAA score (1 degree of freedom, after removing from the analyses the MedDiet+nuts group) was also statistically significant in the most adjusted model ($p = 0.013$). We repeated the analyses additionally adjusting for quartiles of an acylcarnitine score and the results did not materially change (data not shown).

Figure 2 shows the HR of incident type 2 diabetes across quartiles of baseline levels of each plasma amino acid. Each of the BCAAs and tyrosine was associated with a higher risk of incident type 2 diabetes, with significant linear dose-response trends. The weakest association was observed for phenylalanine and the strongest for leucine.

Effects of dietary intervention on BCAAs/AAAs and type 2 diabetes Figure 3 shows the HRs for the joint effects of the intervention and the baseline plasma levels of the BCAA and AAA scores (dichotomised at their median) on the risk of type 2 diabetes. In the BCAA score, the highest risk was found in the control group when baseline levels of BCAA were higher than the median, with HR 2.04 (95% CI 1.29, 3.23) compared with the control group with baseline BCAA score below the median. A negative and significant association was found in the MedDiet+EVOO with baseline score below the median, both in the BCAA and AAA scores.

The intervention with MedDiet+EVOO was associated with significant reductions in the average levels of the BCAA score after one year, not only with respect to baseline levels, but also in comparison with the control group ($p = 0.005$; Fig. 4). Changes in individual amino acids according to intervention group are presented in ESM Fig. 1. After one year, the intervention with MedDiet-EVOO was associated with significant reductions in leucine and isoleucine.

Table 1 Baseline participant characteristics according to diabetic status and baseline scores of metabolites

	By diabetes incidence		By extreme quartiles of BCAA		By extreme quartiles of AAA	
	Subcohort[a]	Cases	Q1	Q4	Q1	Q4
n	694	251	194	254	222	239
Age (years)	66.5 (5.7)	66.4 (5.7)	67.9 (5.4)	65.2 (5.7)	66.5 (5.8)	66.3 (5.6)
Sex (% women)	62.8	55.0	89.7	37.8	63.1	57.7
Intervention group, %						
MedDiet+EVOO	30.7	29.9	30.9	32.3	33.3	30.1
MedDiet+nuts	37.2	33.9	38.7	36.2	36.0	37.2
Control	32.1	36.3	30.4	31.5	30.6	32.6
Hypertension, %	90.8	96.0	91.2	92.1	90.1	91.6
Dyslipidaemia, %	85.0	79.7	88.7	80.7	83.3	84.1
Smoking, %						
Never	61.0	52.6	78.4	43.7	59.5	59.4
Former	22.6	22.3	11.34	28.4	23.0	22.6
Current	16.4	25.1	10.31	28.0	17.6	18.0
Waist circumference, cm	99.0 (10.7)	103.4 (10.0)	96.7 (10.5)	102.6 (9.2)	98.7 (10.4)	102.4 (10.2)
BMI, kg/m^2	29.9 (3.6)	30.8 (3.3)	29.7 (3.8)	30.5 (3.2)	29.5 (3.6)	30.7 (3.6)
Physical activity, MET-min/day	238 (238)	249 (232)	206 (195)	253 (251)	258 (228)	231 (255)
Education, %						
Elementary or lower	75.4	76.5	82.5	69.7	75.7	72.0
Secondary or higher	24.6	23.5	17.5	30.3	24.3	28.0
Total energy intake, MJ/day	9.53 (2.37)	9.74 (2.60)	8.99 (2.03)	10.01 (2.59)	9.66 (2.35)	9.67 (2.45)
Mediterranean diet[b]	8.6 (1.9)	8.5 (1.8)	8.6 (1.9)	8.5 (1.9)	8.6 (1.9)	8.4 (1.9)
Fasting glucose, mmol/l	5.5 (0.8)	6.5 (1.0)	5.6 (1.0)	6.0 (0.9)	5.8 (1.0)	5.8 (1.0)
Fasting insulin, pmol/l[c]	99.0 (48.8)	119.6 (68.6)	82.2 (38.4)	125.0 (59.7)	89.5 (47.8)	121.1 (65.8)
HOMA-IR index[c]	3.6 (2.1)	5.1 (3.1)	3.0 (1.7)	4.8 (2.9)	3.4 (2.2)	4.7 (2.9)

Values are mean (SD) or percentages

BCAA score was calculated as a sum of levels at baseline of leucine, valine and isoleucine

AAA score was calculated as a sum of levels at baseline of phenylalanine and tyrosine

[a] 53 cases are included in the randomly selected subcohort

[b] This score is based on the 14-item PREDIMED screener of adherence to the Mediterranean diet

[c] Available only from 572 participants in the subcohort and 176 cases

When we additionally adjusted for changes in HOMA-IR or in insulin at 1 year (ESM Table 1), we observed that, after one year, the intervention with the MedDiet+EVOO brought about average reductions in SD of −0.21 (95% CI −0.37, −0.05) and −0.23 (95% CI −0.40, −0.07) in the overall BCAA score after adjusting for HOMA-IR and insulin, respectively. These reductions were also statistically significant for each of the three individual BCAAs.

We found no effect of the interventions on changes in plasma insulin after 1 year, with average changes in plasma insulin of −0.97 (95% CI −10.92, 8.92) pmol/l and 2.76 (95% CI −7.22, 12.75) pmol/l, for MedDiet+EVOO and MedDiet+nuts, respectively. Similarly, no significant effects were found for changes in the HOMA-IR index after 1 year. The adjusted mean changes after 1 year of intervention were −0.26 (95% CI −0.74, 0.21) for participants in the MedDiet+EVOO group and −0.07 (95% CI −0.56, 0.41) for participants in the MedDiet+nuts group.

We examined whether changes in amino acids after 1 year of intervention were related with the subsequent incidence of type 2 diabetes occurring after one year (Table 3). In the overall sample, only for isoleucine was the increase after 1 year positively associated with the risk of type 2 diabetes. However, these analyses were conducted with only cases occurring after the first year ($n = 158$) and may have limited statistical power. We observed positive associations in the point estimates, but with wider confidence intervals than for baseline levels. We found some evidence suggesting that the associations between changes in amino acids after 1 year and type 2 diabetes were significantly stronger in the control rather than in the intervention groups. Increases in isoleucine and in the BCAA score after 1 year were positively associated with

Table 2 Incident type 2 diabetes according to baseline plasma branched-chain and aromatic amino acid scores in the PREDIMED trial, 2003–2010

	Overall	*p* value	MedDiet+EVOO	*p* value	MedDiet+nuts	*p* value	Control group	*p* value	*p* for interaction[a]
Subcohort/cases	694[b]/251		213/75		258/85		223/91		
Amino acids, mean[c] (SD), μmol/l									
BCAAs	409.0 (77.3)		410.6 (83.4)		410.1 (76.7)		406.3 (71.8)		
AAAs	111.0 (24.2)		111.0 (23.8)		112.0 (28.3)		109.9 (19.1)		
Model adjustments, HR (95% CI)									
Model 1[d], per 1 SD									
BCAAs[e]	1.54 (1.33, 1.80)	<0.001	1.86 (1.42, 2.44)	0.007	0.96 (0.72, 1.29)	0.809	2.06 (1.56, 2.73)	<0.001	0.007
AAAs[e]	1.18 (1.03, 1.35)	0.016	1.28 (1.01, 1.60)	0.038	0.89 (0.70, 1.14)	0.344	1.48 (1.14, 1.92)	0.003	0.005
Model 2[f], per 1 SD									
BCAAs	1.48 (1.27, 1.73)	<0.001	1.70 (1.27, 2.26)	0.041	0.95 (0.70, 1.29)	0.739	2.14 (1.61, 2.83)	<0.001	0.017
AAAs	1.12 (0.97, 1.29)	0.110	1.15 (0.90, 1.48)	0.268	0.88 (0.68, 1.14)	0.339	1.46 (1.12, 1.91)	0.005	0.065
Model 3[g], per 1 SD									
BCAAs	1.62 (1.37, 1.91)	<0.001	2.03 (1.49, 2.78)	<0.001	0.83 (0.57, 1.21)	0.344	2.32 (1.69, 3.18)	<0.001	<0.001
AAAs	1.22 (1.05, 1.41)	0.008	1.47 (1.08, 2.01)	0.015	0.94 (0.68, 1.28)	0.677	1.38 (1.06, 1.81)	0.018	0.048

Data are mean (SD) and HR (95% CI)

[a] *p* for interaction with 2 degrees of freedom: BCAA score (or AAA score) × MedDiet+EVOO and BCAA score (or AAA score) × MedDiet+nuts

[b] 53 cases were included in the randomly selected subcohort ($n = 694$)

[c] Mean and SD calculated with the absolute values of leucine, isoleucine, valine, phenylalanine and tyrosine

The means presented in the table correspond to all participants

For the subcohort ($n = 694$), the means (SD) for BCAAs and AAAs were 403.3 (75.5) and 111.0 (22.8), respectively, for the three groups together

[d] Model 1: Adjusted for age (years), sex (male, female) and intervention group (MedDiet+EVOO, MedDiet+nuts) and stratified by recruitment centre

[e] An inverse normal transformation was applied to raw values for leucine, isoleucine and valine or phenylalanine and tyrosine and a sum of these values was computed to calculate the BCAA or AAA score, respectively

[f] Model 2: Adjusted as for Model 1, plus BMI (kg/m^2), smoking (never/current/former), leisure-time physical activity (MET-min/day), dyslipidaemia and hypertension

[g] Model 3: Adjusted as for Model 2, plus baseline fasting glucose (mean + quadratic term of centred mean)

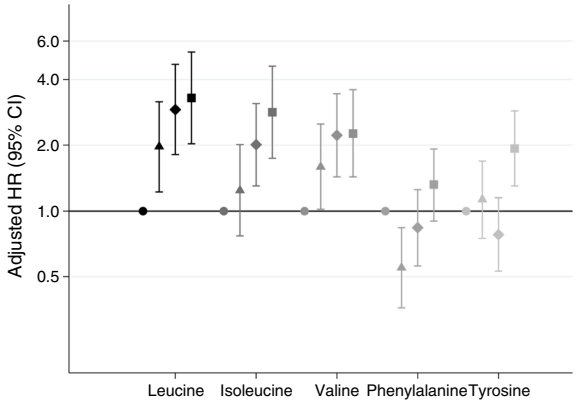

Fig. 2 HRs (95% CI) for type 2 diabetes by quartiles of baseline plasma amino acid levels. HRs are stratified by recruitment centre and adjusted for age (years), sex (male, female) and intervention group (MedDiet+EVOO, MedDiet+nuts), BMI (kg/m^2), smoking (never/current/former), leisure-time physical activity (MET-min/day), dyslipidaemia, hypertension and mean + quadratic term of baseline plasma glucose (centred on the sample mean). Circles, quartile 1 (reference); triangles, quartile 2; diamonds, quartile 3; squares, quartile 4. *p* values for trend: <0.001 (leucine); <0.001 (isoleucine); 0.002 (valine); 0.181 (phenylalanine); 0.004 (tyrosine). The *y*-axis is on a log scale

higher risk of type 2 diabetes only in the control group, but not in the two MedDiet groups (*p* for the interaction <0.001).

When we categorised individual metabolites according to levels of change (decrease/no change/increase), we observed a higher risk of type 2 diabetes in participants with increases after 1 year in comparison with those showing no relevant changes (less than 1 SD) for isoleucine and in the overall BCAA score, with HRs of 1.88 (95% CI 1.20, 2.96) and 2.01 (95% CI 1.27, 3.18), respectively (ESM Table 2). We also observed a lower risk of type 2 diabetes in participants with decreases in phenylalanine after 1 year in comparison

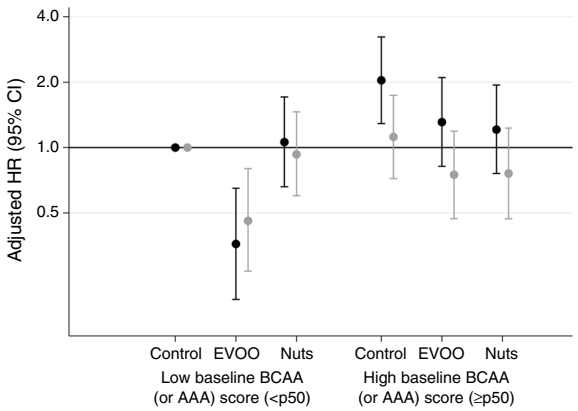

Fig. 3 Joint effect of MedDiet (MedDiet+EVOO, MedDiet+nuts) and baseline BCAA and AAA scores, adjusted for age (years), sex (male, female), BMI (kg/m^2), smoking (never/current/former), leisure-time physical activity (MET-min/day), dyslipidaemia, hypertension, baseline fasting glucose (mean + quadratic term of centred mean) and stratified by recruitment centre. BCAA, black; AAA, grey. Control groups with low BCAA or AAA are reference groups. p50, 50th percentile. The *y*-axis is on a log scale

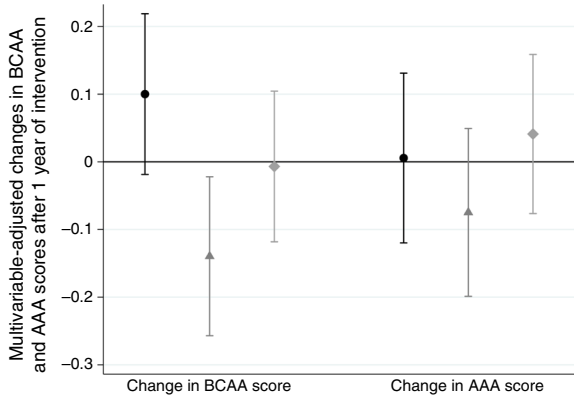

Fig. 4 Changes in BCAA and AAA scores after 1 year of intervention, by intervention group, adjusted for age (years), sex (male, female), BMI (kg/m^2), smoking (never/current/former), leisure-time physical activity (MET-min/day), dyslipidaemia, hypertension, baseline fasting glucose and baseline BCAA (or AAA) score. Circles, control; triangles, MedDiet+EVOO; diamonds, MedDiet+nuts

with participants with no relevant changes (HR 0.55 [95% CI 0.33, 0.93]).

Finally, increases in HOMA-IR after 1 year were positively associated with both BCAA and AAA scores (ESM Table 3). These associations were stronger in the control group than in MedDiet groups, but no significant interactions were observed between amino acid levels and intervention groups on HOMA-IR changes (*p* for interaction = 0.246 for BCAA score and 0.754 for AAA score). Correlations between changes in BCAA or in AAA scores after 1 year and increases in HOMA-IR after 1 year were 0.24 ($p < 0.001$) and 0.19 ($p < 0.001$), respectively.

Discussion

We observed that: (1) Baseline BCAA and AAA scores were associated with a higher risk of incident type 2 diabetes; (2) the intervention with the MedDiet+EVOO was inversely associated with type 2 diabetes in participants with lower baseline BCAA and AAA values (below the median); (3) increases in BCAAs after 1 year was associated with a higher risk of subsequently developing type 2 diabetes (during years 2 to 7 of follow-up) only in the control group, but not in the active intervention groups, of the trial, with statistically significant interactions and (4) the intervention with the MedDiet+EVOO was associated with significant reductions in the overall BCAA score after 1 year.

These findings suggest that a Mediterranean diet could mitigate the adverse effects of elevated plasma levels of BCAA and AAA on type 2 diabetes risk. Of particular interest, the MedDiet+EVOO was associated with lower risk of diabetes in participants with low baseline levels of BCAA and AAA and it was also able to reduce circulating levels of BCAA after 1 year. These findings may explain in part our

Table 3 Associations between increases (per SD) in amino acid levels after 1 year with the risk of incident type 2 diabetes; the PREDIMED trial, 2003–2010

	Overall		MedDiet+EVOO		MedDiet+nuts		Control group		*p* for interaction[a]
	SD[b]	HR per SD[c] (95% CI)	SD[b]	HR per SD[c] (95% CI)	SD[b]	HR per SD[c] (95% CI)	SD[b]	HR per SD[c] (95% CI)	
Subcohort /cases	541[d]/158		176/48		200/52		165/58		
Amino acids									
Leucine	24.0	1.17 (0.94, 1.45)	25.2	1.15 (0.71, 1.87)	24.9	1.33 (0.84, 2.10)	21.5	1.59 (0.99, 2.57)	<0.001
Isoleucine	12.3	1.27 (1.03, 1.57)	12.7	1.48 (0.93, 2.35)	12.8	1.15 (0.72, 1.83)	11.1	1.75 (1.11, 2.76)	0.002
Valine	35.9	1.11 (0.90, 1.38)	36.3	0.78 (0.48, 1.26)	36.9	1.32 (0.86, 2.03)	34.3	1.53 (0.98, 2.37)	<0.001
Phenylalanine	7.1	1.03 (0.85, 1.26)	7.5	0.89 (0.56, 1.42)	7.0	1.14 (0.72, 1.79)	6.6	1.17 (0.80, 1.72)	0.045
Tyrosine	15.9	1.19 (0.97, 1.45)	15.3	0.91 (0.61, 1.37)	18.9	2.01 (1.11, 3.66)	12.5	1.11 (0.74, 1.66)	0.173
BCAA score	68.7	1.18 (0.95, 1.47)	70.5	1.01 (0.61, 1.69)	71.0	1.13 (0.84, 2.03)	63.5	1.61 (1.02, 2.54)	<0.001
AAA score	19.6	1.08 (0.89, 1.30)	19.5	0.92 (0.59, 1.42)	22.8	1.26 (0.79, 2.01)	15.8	1.17 (0.80, 1.72)	0.045

[a] *p* for interaction with two interaction terms (2 degrees of freedom): BCAA score (cont.) × MedDiet+EVOO and BCAA score (cont.) × MedDiet+nuts

[b] SD of changes at 1 year were calculated based on the of the absolute values of the individual metabolites (μmol/l)

[c] An inverse normal transformation was applied to raw values. Model adjusted for metabolite (or score) level at baseline, age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), BMI (kg/m2), smoking (never/current/former), leisure-time physical activity (MET-min/day), dyslipidaemia, hypertension, baseline fasting glucose (mean + quadratic term of centred mean) and stratified by recruitment centre

[d] 36 cases were included in the randomly selected subcohort

previous finding that, among participants of PREDIMED who were initially free of diabetes, the MedDiet+EVOO intervention significantly reduced risk of type 2 diabetes [16]. Our results are consistent with the findings from our systematic review on metabolomics and type 2 diabetes [6], other recent findings [5, 7–12, 25] and our previous observation on the association between plasma levels of BCAA and incident cardiovascular disease [26].

The most likely metabolic mechanism to explain the observed associations is related to the activation by amino acids of the mammalian target of rapamycin (mTOR)/S6 kinase 1 pathway [27]. Mendelian randomisation analyses have suggested both the causal role of BCAA metabolism in the aetiology of type 2 diabetes [25] but also that higher BCAA levels are not likely to be a cause, but rather a consequence, of insulin resistance [28]. Therefore, debate still persists on whether the BCAAs are actually causal factors for the development of insulin resistance or merely fellow travellers, which nevertheless can be used as clinically useful biomarkers [1].

Elevated levels of BCAAs are known to activate mTOR complex 1 (mTORC1) which leads to insulin resistance through the phosphorylation of insulin receptor substrate 1 (IRS-1) [1, 29]. BCAAs stimulate the activation of the redox-sensitive transcription factor NF-κB, resulting in the release of pro-inflammatory molecules (interleukin-6, tumour necrosis factor-α, intracellular adhesion molecule-1, CD40L) and the migration of peripheral mononuclear blood cells [27]. These pro-inflammatory changes could contribute to the development of insulin resistance. Furthermore, in mouse models, 3-hydroxyisobutyrate (identified as a catabolic intermediate of valine) acts as a paracrine regulator of transendothelial fatty acid transport by activating the endothelial transport of fatty acids and the uptake of these fatty acids, thus leading to lipid accumulation in muscle and consequently to insulin resistance [30].

In large prospective epidemiologic studies, a higher intake of BCAAs has been significantly associated with a higher subsequent risk of developing type 2 diabetes [31]. Randomised dietary interventions (in weight-loss trials) showed that decreases in plasma tyrosine were associated with improvements in insulin resistance independent of weight loss [32]. This evidence, together with parallel results in obese children [33] and evidence on BCAA-associated metabolic disorders in elderly participants [34], supports a causal role for BCAAs in the development of insulin resistance and type 2 diabetes, independently of weight change.

The present findings, based on a unique longitudinal assessment with repeated measurements and a randomised intervention, shed light on a potential role of BCAAs/AAAs in the development of type 2 diabetes and the benefits of a high-quality dietary pattern to modulate their adverse effects. In fact, we showed for the first time the ability of an extra-virgin olive oil-rich Mediterranean diet to decrease the levels of plasma BCAA in a randomised trial. These associations persisted after additionally adjusting for changes in insulin or HOMA-IR and this finding suggests that the effect of the MedDiet on BCAA levels is not likely to be importantly mediated by changes in plasma insulin or in HOMA-IR.

The strengths of our study include adjustment for multiple potential confounders within a well-characterised trial, together with the design of a case-cohort study, which retains randomisation, maximises the efficiency of a high-throughput metabolomic profiling and enables the extension of our results to the full cohort. Several limitations also deserve consideration. First, type 2 diabetes was a secondary endpoint and not the primary endpoint of the PREDIMED trial. Second, our results may not be generalisable to other populations because all the study participants lived in a Mediterranean country and were at high cardiovascular risk. Third, we cannot rule out residual confounding in our observational associations between BCAAs/AAAs (or their changes) and the risk of type 2 diabetes.

In conclusion, elevated baseline levels of BCAA and AAA as well as increase in these amino acids after 1 year were associated with higher risk of type 2 diabetes in a Mediterranean population at high cardiovascular risk. A Mediterranean diet supplemented with EVOO was able to reduce the levels of BCAA and attenuate the positive association between BCAA levels and type 2 diabetes incidence.

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Data availability The datasets generated and analysed during the current study are not publicly available due to national data regulations and for ethical reasons, including the possibility that some information might compromise research participants' consent because our participants only gave their consent for the use of their data by the original team of investigators. However, these data can be requested by signing a data sharing agreement as approved by the relevant research ethics committees and the steering committee of the PREDIMED trial.

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Duality of interest ER has received honoraria for lectures and grants for research through his institution from the California Walnut Commission and is a nonpaid member of its Scientific Advisory Committee. JS-S has received grants for research through his institution from the International Nut and Dried Fruit Council and is a nonpaid member of its Scientific Advisory Committee. The rest of the authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MR-C and MAM-G conducted the statistical analyses and drafted the article. MR-C, FBH, ET, CBC, LL, JS-S, and MAM-G made substantial contributions to the conception and design of the work. All authors contributed substantially in the acquisition of data or analysis and interpretation of data. All authors revised the article critically for important intellectual content. All authors approved the version to be published.

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ORIGINAL ARTICLE

Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts

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ABSTRACT

BACKGROUND

Observational cohort studies and a secondary prevention trial have shown inverse associations between adherence to the Mediterranean diet and cardiovascular risk.

METHODS

In a multicenter trial in Spain, we assigned 7447 participants (55 to 80 years of age, 57% women) who were at high cardiovascular risk, but with no cardiovascular disease at enrollment, to one of three diets: a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with mixed nuts, or a control diet (advice to reduce dietary fat). Participants received quarterly educational sessions and, depending on group assignment, free provision of extra-virgin olive oil, mixed nuts, or small nonfood gifts. The primary end point was a major cardiovascular event (myocardial infarction, stroke, or death from cardiovascular causes). After a median follow-up of 4.8 years, the trial was stopped on the basis of a prespecified interim analysis. In 2013, we reported the results for the primary end point in the *Journal*. We subsequently identified protocol deviations, including enrollment of household members without randomization, assignment to a study group without randomization of some participants at 1 of 11 study sites, and apparent inconsistent use of randomization tables at another site. We have withdrawn our previously published report and now report revised effect estimates based on analyses that do not rely exclusively on the assumption that all the participants were randomly assigned.

RESULTS

A primary end-point event occurred in 288 participants; there were 96 events in the group assigned to a Mediterranean diet with extra-virgin olive oil (3.8%), 83 in the group assigned to a Mediterranean diet with nuts (3.4%), and 109 in the control group (4.4%). In the intention-to-treat analysis including all the participants and adjusting for baseline characteristics and propensity scores, the hazard ratio was 0.69 (95% confidence interval [CI], 0.53 to 0.91) for a Mediterranean diet with extra-virgin olive oil and 0.72 (95% CI, 0.54 to 0.95) for a Mediterranean diet with nuts, as compared with the control diet. Results were similar after the omission of 1588 participants whose study-group assignments were known or suspected to have departed from the protocol.

CONCLUSIONS

In this study involving persons at high cardiovascular risk, the incidence of major cardiovascular events was lower among those assigned to a Mediterranean diet supplemented with extra-virgin olive oil or nuts than among those assigned to a reduced-fat diet. (Funded by Instituto de Salud Carlos III, Spanish Ministry of Health, and others; Current Controlled Trials number, ISRCTN35739639.)

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*The PREDIMED study investigators are listed in the Supplementary Appendix, available at NEJM.org.

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The traditional Mediterranean diet is characterized by a high intake of olive oil, fruit, nuts, vegetables, and cereals; a moderate intake of fish and poultry; a low intake of dairy products, red meat, processed meats, and sweets; and wine in moderation, consumed with meals.[1] In observational cohort studies[2,3] and a secondary prevention trial (the Lyon Diet Heart Study),[4] increasing adherence to the Mediterranean diet has been consistently associated with lower cardiovascular risk.[2-4] A systematic review ranked the Mediterranean diet as the most likely dietary model to provide protection against coronary heart disease.[5] Small clinical trials have uncovered plausible biologic mechanisms to explain the salutary effects of this food pattern.[6-9] We designed a randomized trial, PREDIMED (Prevención con Dieta Mediterránea), to test the efficacy of two Mediterranean diets (one supplemented with extra-virgin olive oil and another with nuts), as compared with a control diet (advice on a low-fat diet), on primary cardiovascular prevention. Our original report was published in the *Journal* in 2013.[10] A 2017 analysis[11] of the distributions of baseline variables in randomized trials identified the PREDIMED trial as having distributions that were significantly different from what would have been expected to result from randomization. This report led to our conducting a review of how participants were assigned to various intervention groups; that review revealed irregularities in our randomization procedures. Therefore, we have withdrawn our original report[12] and now publish a new report.

We describe the protocol deviations and report revised intention-to-treat and per-protocol effect estimates that do not rely exclusively on the assumption that all the participants had been randomly assigned to the intervention groups. A detailed description of the changes that have been introduced and departures from the protocol is provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Methods

Study Design

The PREDIMED study was designed as a parallel-group, multicenter, randomized trial. Details of the study design have been reported previously.[13,14] The protocol, available at NEJM.org, was approved by the institutional review boards at all study locations. The authors vouch for the accuracy and completeness of the data and all analyses and attest that this report accurately describes the conduct of the study as we know it.

Funding was provided by grants from Instituto de Salud Carlos III, Spanish Ministry of Health. Supplemental foods were donated, including extra-virgin olive oil (by Hojiblanca and Patrimonio Comunal Olivarero, both in Spain), walnuts (by the California Walnut Commission), almonds (by Borges, in Spain), and hazelnuts (by Morella Nuts, in Spain). None of the sponsors had any role in the study design, data analysis, or reporting of the results.

Participant Selection and Randomization

Eligible participants were men (55 to 80 years of age) or women (60 to 80 years of age) with no cardiovascular disease at enrollment, who had either type 2 diabetes mellitus or at least three of the following major risk factors: smoking, hypertension, elevated low-density lipoprotein cholesterol levels, low high-density lipoprotein cholesterol levels, overweight or obesity, or a family history of premature coronary heart disease. Detailed enrollment criteria are provided on pages 18 and 19 in the Supplementary Appendix. All the participants provided written informed consent.

The protocol specified that participants were to be randomly assigned, in a 1:1:1 ratio, to one of three dietary intervention groups: a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with nuts, or a control diet. Enrollment began on June 25, 2003, and the last participant was recruited on June 30, 2009. The analyses in this report were based on a database locked as of September 2011 and included primary end-point events occurring through December 1, 2010. Randomization was concealed with the use of closed envelopes[8] during part of the pilot phase of the study, but envelopes were not used for the remainder of the study. A computer-generated random-number sequence provided randomization tables for the 11 participating sites, which encompassed 169 clinics. These tables included four strata (men <70 years of age, men ≥70 years of age, women <70 years of age, and women ≥70 years of age) and were initially generated for 1000 participants (250 per stratum) for each site. We did not use blocks for randomization. Further details on the use of these tables at each of the 11 sites can

be found on pages 8 and 9 and 78 through 82 in the Supplementary Appendix. All the sites were given the same randomization sequence.

There were departures from the randomization procedures that had been specified in the protocol that were not described in our original report. We included 425 participants who shared a household with a previously enrolled participant. These 425 participants were not randomly assigned but were assigned to the same intervention as the member of the household who was already enrolled (Tables S2 and S3 in the Supplementary Appendix). This was done to allow the recruitment of eligible household members and to avoid members of the same household being assigned to different diets. After the study had begun, the steering committee approved this protocol change. The protocol was not amended, and this protocol change was not described in the original report published in the *Journal*. In July 2017, we learned that at 1 of the 11 study sites (Site D), 467 participants were not randomly assigned as individual participants but instead were assigned according to clinic — that is, all the participants in each clinic received the same intervention (2 clinics assigned a Mediterranean diet with extra-virgin olive oil, 5 assigned a Mediterranean diet with nuts, and 4 assigned a control diet) (see pages 9 and 10 in the Supplementary Appendix for additional details). In addition, review of the documentation about randomization procedures and of the actual assignments to the three groups suggested that the randomization tables were inconsistently used in another study site (Site B, 593 participants) (see pages 10 and 11 in the Supplementary Appendix for details).

INTERVENTIONS AND MEASUREMENTS

The dietary intervention[8,13-15] is detailed on pages 20 through 24 in the Supplementary Appendix. The specific recommended diets are summarized in Table 1. Participants in the group assigned to a Mediterranean diet with extra-virgin olive oil received 1 liter of the oil per week per household, with the recommendation to consume at least 4 tablespoons per day of extra-virgin olive oil per person. Participants in the group assigned to a Mediterranean diet with nuts received 30 g of mixed nuts per day per person (15 g of walnuts, 7.5 g of hazelnuts, and 7.5 g of almonds) at no cost, and those in the control group received small nonfood gifts. No total calorie restriction was advised, nor was physical activity promoted.

Table 1. Summary of Dietary Recommendations to Participants in the Mediterranean-Diet Groups and the Control-Diet Group.

Food	Goal
Mediterranean diet	
Recommended	
Olive oil*	≥4 tbsp/day
Tree nuts and peanuts†	≥3 servings/wk
Fresh fruits	≥3 servings/day
Vegetables	≥2 servings/day
Fish (especially fatty fish), seafood	≥3 servings/wk
Legumes	≥3 servings/wk
Sofrito‡	≥2 servings/wk
White meat	Instead of red meat
Wine with meals (optionally, only for habitual drinkers)	≥7 glasses/wk
Discouraged	
Soda drinks	<1 drink/day
Commercial bakery goods, sweets, and pastries§	<2 servings/wk
Spread fats	<1 serving/day
Red and processed meats	<1 serving/day
Low-fat diet (control)¶	
Recommended	
Low-fat dairy products	≥3 servings/day
Bread, potatoes, pasta, rice	≥3 servings/day
Fresh fruits	≥3 servings/day
Vegetables	≥2 servings/day
Lean fish and seafood	≥3 servings/wk
Discouraged	
Vegetable oils (including olive oil)	≤2 tbsp/day
Commercial bakery goods, sweets, and pastries§	≤1 serving/wk
Nuts and fried snacks	≤1 serving/wk
Red and processed fatty meats	≤1 serving/wk
Visible fat in meats and soups‖	Always remove
Fatty fish, seafood canned in oil	≤1 serving/wk
Spread fats	≤1 serving/wk
Sofrito‡	≤2 servings/wk

* The amount of olive oil includes oil used for cooking and salads and oil consumed in meals eaten outside the home. In the group assigned to the Mediterranean diet with extra-virgin olive oil, the goal was to consume 50 g (approximately 4 tbsp) or more per day of the polyphenol-rich olive oil supplied, instead of the ordinary refined variety, which is poor in polyphenols. The participants received a free supply (15 liters every 3 months) to include the oil used for cooking and family needs.

† For participants assigned to the Mediterranean diet with nuts, the recommended consumption was one daily serving (30 g, composed of 15 g of walnuts, 7.5 g of almonds, and 7.5 g of hazelnuts). Participants received for free the needed allotments of tree nuts in packages of 2 kg of walnuts, 1 kg of almonds, and 1 kg of hazelnuts every 3 months, with the extra amounts to be shared with family members.

‡ Sofrito is a sauce made with tomato and onion, often including garlic and aromatic herbs, and slowly simmered with olive oil.

§ Commercial bakery goods, sweets, and pastries (not homemade) included cakes, cookies, biscuits, and custard.

¶ Up to September 2006, a brief personal recommendation and a leaflet with written guidelines to attain these goals (see page 53 in the Supplementary Appendix) were given to participants on a yearly basis. Starting in October 2006, the intensity of these recommendations was increased, including also group sessions and personal advice repeated every 3 months (i.e., with the same intensity and frequency of contacts as in the two Mediterranean-diet groups). The composition of the recommended diet, however, was not changed.

‖ Participants were advised to remove the visible fat (or the skin) of chicken, duck, pork, lamb, or veal before cooking and the fat of soups, broths, and cooked meat dishes before consumption.

For participants in the two Mediterranean-diet groups, dietitians held individual and group dietary-training sessions at the baseline visit and quarterly thereafter. In each session, participants completed a 14-item dietary questionnaire to assess adherence to the Mediterranean diet[8,16] (Table S4 in the Supplementary Appendix) so that personalized advice could be provided to the study participants in these groups. Questionnaire scores ranged from 0 to 14, with scores lower than 10 defined as low adherence to the Mediterranean diet.

Participants in the control group also received dietary training at the baseline visit and completed the 14-item questionnaire at baseline to assess their adherence to the Mediterranean diet. During the first 3 years of the study, they received a leaflet explaining the low-fat diet (see page 53 in the Supplementary Appendix) on a yearly basis. However, the realization that the more infrequent visit schedule and less intense support for the control group might be limitations of the study prompted us to amend the protocol in October 2006. Thereafter, participants who were assigned to the control diet received personalized advice and were invited to group sessions with the same frequency and intensity as those in the Mediterranean-diet groups, with the use of a separate 9-item dietary questionnaire (Table S5 in the Supplementary Appendix). Scores ranged from 0 to 9, with higher scores indicating greater adherence to a low-fat diet. Except for the Site D clinics discussed above and 11 clinics at Site I, all clinics of sufficient size delivered all three of the interventions (see page 11 in the Supplementary Appendix).

A general medical questionnaire, a 137-item validated food-frequency questionnaire,[17] and the Minnesota Leisure-Time Physical Activity Questionnaire were administered on a yearly basis.[13] Information from the food-frequency questionnaire was used to estimate intake of energy and nutrients. Weight, height, and waist circumference were directly measured annually.[18] Biomarkers of adherence, including urinary hydroxytyrosol levels (to confirm adherence in the group receiving extra-virgin olive oil) and plasma alpha-linolenic acid levels (to confirm adherence in the group receiving mixed nuts), were measured in random subsamples of participants at 1, 3, and 5 years (Figs. S9 and S10 in the Supplementary Appendix).

END POINTS

The primary end point was a composite of myocardial infarction, stroke, and death from cardiovascular causes. Secondary end points were stroke, myocardial infarction, death from cardiovascular causes, and death from any cause. We used four sources of information to identify end points: repeated contacts with participants, contacts with family physicians, a yearly review of medical records, and consultation of the National Death Index. All medical records that were related to end points were examined by the end-point adjudication committee, whose members were unaware of the intervention-group assignments. Only end points that were confirmed by the adjudication committee and that occurred between June 25, 2003, and December 1, 2010, were included in the analyses. The criteria for adjudicating primary and secondary end points are detailed on pages 26 and 27 in the Supplementary Appendix.

STATISTICAL ANALYSIS

We initially estimated that a sample of 9000 participants would be required to provide a statistical power of 80% to detect a 20% lower risk of the primary end point in each Mediterranean-diet group than in the control-diet group during a 4-year follow-up period, assuming an absolute cumulative risk of 12% in the control group.[13,19] In April 2008, on the advice of the data and safety monitoring board and on the basis of lower-than-expected rates of end-point events, the sample size was recalculated as 7400 participants, with the assumption of a 6-year follow-up period because of slower-than-expected recruitment and an underlying absolute cumulative risk of the primary end point of 8.8% in the control group and 6.6% in the Mediterranean-diet groups. The relationships between enrollment size and statistical power, under several assumptions, are shown in Figure S6 in the Supplementary Appendix.

Yearly interim analyses began on March 2008 after a median of 2 years of follow-up. With the use of O'Brien–Fleming stopping boundaries, the P values for stopping the study at each yearly interim analysis were 5×10^{-6}, 0.001, 0.009, and 0.02 for benefit and 9×10^{-5}, 0.005, 0.02, and 0.05 for adverse effects.[20] The stopping boundary for the benefit of the Mediterranean diets with re-

spect to the primary end point was crossed at the fourth interim evaluation; on July 22, 2011, the data and safety monitoring board recommended stopping the study on the basis of end points documented through December 1, 2010. After the study was stopped, we advised all the participants, including those in the control group, to follow the Mediterranean diet.

The interim and original primary analyses estimated differences between the groups assigned to different interventions (intention-to-treat analyses). The information on protocol deviations was not considered in these analyses. Participants were followed from the baseline visit until the occurrence of a primary end-point event, death, or the last contact date from either medical records or study visits. We did not record the date of randomization and thus do not report the time between randomization and the baseline visit; for all the participants, we used the date of the baseline visit as time 0 in our analyses. No participant had a primary or secondary end-point event between randomization and baseline according to our review of the medical records.

We constructed Kaplan–Meier cumulative-incidence curves according to intervention group and calculated hazard ratios on an intention-to-treat basis, with the control group as the reference, using a Cox model with indicators for the Mediterranean diet with extra-virgin olive oil and the Mediterranean diet with nuts. We used robust variance estimators to account for intracluster correlations in all Cox models, considering as clusters the members of the same household and the participants in the same clinic of Site D. We compared baseline characteristics across the three groups and conducted analyses that did not rely on the assumption that all the participants were randomly assigned and that randomization would distribute baseline characteristics of the participants equally across intervention groups. Our main analysis was a multivariable model stratified according to site, sex, and educational level (five categories); to account for potential imbalances in baseline risk factors among the intervention groups, the model included nine other baseline variables as covariates (see page 12 in the Supplementary Appendix). This model was also adjusted for propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups (detailed on pages 12 through 17 in the Supplementary Appendix).

Prespecified subgroup analyses were conducted according to sex, age, body-mass index (BMI), status with respect to cardiovascular risk factors, and baseline adherence to the Mediterranean diet. In sensitivity analyses, we excluded the 1588 participants whose randomization procedures were known or suspected to have deviated from the protocol: all 652 participants from Site D (35 were second members of a household), 593 participants from Site B (47 were second members of a household), and another 343 second household members from other sites. In addition, we performed sensitivity analyses to assess how strong and prevalent an unmeasured confounder would have to be to explain the observed results (Table S25 in the Supplementary Appendix). We also adjusted for missing data and loss to follow-up, implemented other exclusions, and used alternative analytic approaches (see pages 30 through 35 and Figs. S2 and S4 in the Supplementary Appendix).

A secondary analysis estimated the per-protocol effect[21] of the Mediterranean diet as compared with the control diet that would have been observed if all the participants had adhered to their assigned interventions throughout the follow-up period. For participants assigned to the Mediterranean-diet groups, adherence was defined as a score of 10 or higher on the 14-item questionnaire. For those assigned to the low-fat diet, adherence was defined as a fat intake of 30% or less of total energy intake according to the food-frequency questionnaires that were administered annually to the three groups or a score or 6 or higher on the 9-item questionnaire. We censored data for participants when they first stopped adhering to their assigned intervention, estimated inverse-probability weights to adjust for postrandomization prognostic factors, and estimated the hazard ratio for an end-point event in the Mediterranean-diet groups as compared with the low-fat diet group.[22,23]

The validity of the per-protocol effect estimate relies on several assumptions. It assumes that loss to follow-up, data collection, and adherence can be treated as sequentially randomized at each time point, given the measured prognostic factors before and after randomization.[22] Both Mediterranean-diet groups were combined for

precision because only 39% of the events remained uncensored after the application of our strict definition of adherence. We used the predicted values from this model after adding a product term between intervention and time to estimate cumulative-incidence curves (see pages 36 through 38 in the Supplementary Appendix for details).

RESULTS

BASELINE CHARACTERISTICS

From June 25, 2003, through June 30, 2009, a total of 8713 candidates were screened for eligibility, and 7447 were assigned to one of the three intervention groups (Fig. S7 in the Supplementary Appendix). Their baseline characteristics according to intervention group are shown in Table 2, and in Table S23 in the Supplementary Appendix. The exclusion of participants whose randomization procedures were known to have deviated from the protocol did not materially change these results. Drug-treatment regimens at baseline were similar for participants in the three groups, and they continued to be balanced during the follow-up period (Table S6 in the Supplementary Appendix).

Participants were followed for a median of 4.8 years (interquartile range, 2.8 to 5.8). After the baseline visit, 210 participants (2.8%) chose not to attend subsequent visits (1.2% of the participants assigned to a Mediterranean diet with extra-virgin olive oil, 2.7% of those assigned to a Mediterranean diet with nuts, and 4.7% of those in the control group). The rate of study discontinuation (>2 years since last contact) was 11.3% in the control group and 4.9% in the Mediterranean-diet groups; subsequent follow-up was based on reviews of medical records (Fig. S7 and Table S24 in the Supplementary Appendix). Participants who dropped out of the study were, on average, 1.4 years younger than those who remained in the study and had a higher BMI (the weight in kilograms divided by the square of height in meters) by 0.4, a higher waist-to-height ratio (by 0.01), and a lower score for adherence to the Mediterranean diet (by 1.0 points on the 14-item questionnaire) at baseline.

ADHERENCE TO THE DIETARY INTERVENTION

The scores on the 14-item Mediterranean-diet questionnaire increased over the follow-up period for the participants in the two Mediterranean-diet groups (Table S7 and Fig. S8 in the Supplementary Appendix). There were substantial differences between the Mediterranean-diet groups and the control group in 12 of the 14 items (Table S7 in the Supplementary Appendix). Changes in biomarkers also indicated good adherence to the dietary assignments (Figs. S9 and S10 in the Supplementary Appendix).

Participants in the two Mediterranean-diet groups increased weekly servings of fish (by 0.3 servings) and legumes (by 0.4 servings) in comparison with those in the control group (Table S8 in the Supplementary Appendix). In addition, participants assigned to a Mediterranean diet with extra-virgin olive oil and those assigned to a Mediterranean diet with nuts increased their consumption of extra-virgin olive oil (to 50 and 32 g per day, respectively) and nuts (to 0.9 and 6 servings per week, respectively). The main nutrient changes in the Mediterranean-diet groups reflected the fat content and composition of the supplemental foods (Tables S9 and S10 in the Supplementary Appendix). No relevant diet-related adverse effects were reported (see page 38 in the Supplementary Appendix). We found little difference in changes in physical activity among the three groups.

END POINTS

In the intention-to-treat analysis, there were 96 primary end-point events in the group assigned to a Mediterranean diet with extra-virgin olive oil (3.8%), 83 in the group assigned to a Mediterranean diet with nuts (3.4%), and 109 in the control group (4.4%). The respective incidence rates were 8.1, 8.0, and 11.2 per 1000 person-years, and the 5-year absolute risks were 3.6%, 4.0%, and 5.7%, respectively (Table 3). The unadjusted hazard ratios that used robust variance estimators to account for intracluster correlations were 0.70 (95% confidence interval [CI], 0.53 to 0.92) for a Mediterranean diet with extra-virgin olive oil and 0.70 (95% CI, 0.53 to 0.94) for a Mediterranean diet with nuts as compared with the control diet.

Results of our primary analyses that included adjustment for propensity scores and 12 baseline participant characteristics were similar to those of the unadjusted analyses, with hazard ratios of 0.69 (95% CI, 0.53 to 0.91) for a Mediterranean diet with extra-virgin olive oil and 0.72 (95% CI,

Table 2. Baseline Characteristics of the Participants, According to Intervention Group.*

Characteristic	Mediterranean Diet with EVOO (N=2543)	Mediterranean Diet with Nuts (N=2454)	Control Diet (N=2450)
Female sex — no. (%)†	1493 (58.7)	1326 (54.0)	1463 (59.7)
Age — yr†	67.0±6.2	66.7±6.1	67.3±6.3
Race or ethnic group — no. (%)‡			
White, from Europe	2470 (97.1)	2390 (97.4)	2375 (96.9)
Hispanic, from Central or South America	35 (1.4)	29 (1.2)	38 (1.6)
Other	38 (1.5)	35 (1.4)	37 (1.5)
Smoking status — no. (%)			
Never smoked	1572 (61.8)	1465 (59.7)	1527 (62.3)
Former smoker	618 (24.3)	634 (25.8)	584 (23.8)
Current smoker	353 (13.9)	355 (14.5)	339 (13.8)
Body-mass index†§	29.9±3.7	29.7±3.8	30.2±4.0
Waist circumference — cm	100±10	100±10	101±11
Waist-to-height ratio†¶	0.63±0.06	0.63±0.06	0.63±0.07
Hypertension — no. (%)‖	2088 (82.1)	2024 (82.5)	2050 (83.7)
Type 2 diabetes — no. (%)†**	1282 (50.4)	1143 (46.6)	1189 (48.5)
Dyslipidemia — no. (%)††	1821 (71.6)	1799 (73.3)	1763 (72.0)
Family history of premature CHD — no. (%)‡‡	576 (22.7)	532 (21.7)	560 (22.9)
Medication use — no. (%)			
ACE inhibitors	1236 (48.6)	1223 (49.8)	1216 (49.6)
Diuretics†	534 (21.0)	477 (19.4)	562 (22.9)
Other antihypertensive agents	725 (28.5)	710 (28.9)	758 (30.9)
Statins	1039 (40.9)	964 (39.3)	983 (40.1)
Other lipid-lowering agents	121 (4.8)	145 (5.9)	126 (5.1)
Insulin	124 (4.9)	126 (5.1)	134 (5.5)
Oral hypoglycemic agents†	768 (30.2)	680 (27.7)	757 (30.9)
Antiplatelet therapy	475 (18.7)	490 (20.0)	513 (20.9)
Hormone-replacement therapy§§	42 (2.8)	35 (2.6)	39 (2.7)

* Plus–minus values are means ±SD. Percentages may not total 100 because of rounding. ACE denotes angiotensin-converting enzyme, and EVOO extra-virgin olive oil.
† P<0.05 for comparisons between groups.
‡ Race and ethnic group were determined by the staff of the trial (nurses or dietitians).
§ The body-mass index is the weight in kilograms divided by the square of the height in meters.
¶ The waist-to-height ratio (an index of central obesity) is the waist circumference divided by height.
‖ Hypertension was defined as a systolic blood pressure of 140 mm Hg or higher, a diastolic blood pressure of 90 mm Hg or higher, or the use of antihypertensive therapy.
** Diabetes was defined as a fasting blood glucose level of 126 mg per deciliter (7.0 mmol per liter) or higher on two occasions, a 2-hour plasma glucose level of 200 mg per deciliter (11.1 mmol per liter) or higher during a 75-g oral glucose-tolerance test, or the use of antidiabetic medication.
†† Dyslipidemia was defined as a low-density lipoprotein cholesterol level higher than 160 mg per deciliter (4.1 mmol per liter), a high-density lipoprotein cholesterol level of 40 mg per deciliter (1.0 mmol per liter) or lower in men or 50 mg per deciliter (1.3 mmol per liter) or lower in women, or the use of lipid-lowering therapy.
‡‡ A family history of premature coronary heart disease (CHD) was defined as a diagnosis of the disease in a male first-degree relative younger than 55 years of age or in a female first-degree relative younger than 65 years of age.
§§ The values for hormone-replacement therapy are for women only.

Table 3. Estimates of Cardiovascular Events, According to Intervention Group.*

End Point	Mediterranean Diet with EVOO (N=2543)	Mediterranean Diet with Nuts (N=2454)	Control Diet (N=2450)
No. of person-yr of follow-up	11852	10365	9763
Primary end point†			
No. of events	96	83	109
Incidence rate per 1000 person-yr (95% CI)	8.1 (6.6–9.9)	8.0 (6.4–9.9)	11.2 (9.2–13.5)
5-yr absolute risk — % (95% CI)‡	3.6 (2.8–4.5)	4.0 (3.1–5.0)	5.7 (4.6–6.9)
Secondary end points			
Stroke			
No. of events	49	32	58
Incidence rate per 1000 person-yr (95% CI)	4.1 (3.1–5.5)	3.1 (2.1–4.4)	5.9 (4.5–7.7)
5-yr absolute risk — % (95% CI)	1.7 (1.3–2.4)	1.5 (1.1–2.3)	3.0 (2.3–3.9)
Myocardial infarction			
No. of events	37	31	38
Incidence rate per 1000 person-yr (95% CI)	3.1 (2.2–4.3)	3.0 (2.0–4.2)	3.9 (2.8–5.3)
5-yr absolute risk — % (95% CI)	1.4 (1.0–2.1)	1.6 (1.1–2.3)	2.1 (1.5–2.9)
Death from cardiovascular causes			
No. of events	26	31	30
Incidence rate per 1000 person-yr (95% CI)	2.2 (1.4–3.2)	3.0 (2.0–4.2)	3.1 (2.1–4.4)
5-yr absolute risk — % (95% CI)	1.0 (0.6–1.5)	1.4 (0.9–2.1)	1.6 (1.1–2.3)
Death from any cause			
No. of events	118	116	114
Incidence rate per 1000 person-yr (95% CI)	10.0 (8.2–11.9)	11.2 (9.3–13.4)	11.7 (9.6–14.0)
5-yr absolute risk — % (95% CI)	4.4 (3.6–5.4)	5.4 (4.4–6.6)	5.4 (4.4–6.7)
ITT analysis: hazard ratio for each Mediterranean diet vs. control (95% CI)§			
Primary end point			
Unadjusted	0.70 (0.53–0.92)	0.70 (0.53–0.94)	1.00 (ref)
Adjusted¶	0.69 (0.53–0.91)	0.72 (0.54–0.95)	1.00 (ref)
Secondary end points¶			
Stroke	0.65 (0.44–0.95)	0.54 (0.35–0.82)	1.00 (ref)
Myocardial infarction	0.82 (0.52–1.30)	0.76 (0.47–1.25)	1.00 (ref)
Death from cardiovascular causes	0.62 (0.36–1.06)	1.02 (0.63–1.67)	1.00 (ref)
Death from any cause	0.90 (0.69–1.18)	1.12 (0.86–1.47)	1.00 (ref)
ITT analysis: hazard ratio for Mediterranean diets combined vs. control (95% CI)§			
Primary end point			
Unadjusted	0.70 (0.55–0.89)		1.00 (ref)
Adjusted¶	0.70 (0.55–0.89)		1.00 (ref)
Secondary end points¶			
Stroke	0.58 (0.42–0.82)		1.00 (ref)
Myocardial infarction	0.80 (0.53–1.21)		1.00 (ref)
Death from cardiovascular causes	0.80 (0.51–1.24)		1.00 (ref)
Death from any cause	0.98 (0.77–1.24)		1.00 (ref)

Table 3. (Continued.)

End Point	Mediterranean Diet with EVOO (N=2543)	Mediterranean Diet with Nuts (N=2454)	Control Diet (N=2450)
Primary end point, excluding Site D and second household members‖			
Each Mediterranean diet and control			
No. of participants	2158	2109	2138
5-year absolute risk — % (95% CI)	3.4 (2.6–4.3)	3.9 (3.0–5.0)	5.9 (4.8–7.2)
Hazard ratio (95% CI)¶	0.66 (0.49–0.89)	0.64 (0.47–0.88)	1.00 (ref)
Mediterranean diets combined and control			
5-year absolute risk — % (95% CI)	3.6 (3.0–4.3)		5.9 (4.8–7.2)
Hazard ratio (95% CI)¶	0.65 (0.50–0.85)		1.00 (ref)

* CI denotes confidence interval, and ref reference.
† The primary end point was a composite of myocardial infarction, stroke, and death from cardiovascular causes.
‡ In the combined Mediterranean-diet groups, the 5-year absolute risk of the primary end point was 3.8% (95% CI, 3.2 to 4.4).
§ The intention-to-treat (ITT) analysis included all 7447 participants.
¶ The Cox model was stratified according to sex, recruiting site, and educational level (five categories) and adjusted for age (continuous variable), smoking status (never smoked, former smoker, or current smoker), hypertension at baseline (yes or no), dyslipidemia at baseline (yes or no), diabetes at baseline (yes or no), family history of premature coronary heart disease (yes or no), body-mass index (continuous variable), waist-to-height ratio (continuous variable), physical activity (in quintiles), and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups (see pages 12 through 17 in the Supplementary Appendix). Robust standard errors to account for intracluster correlations were used.
‖ The analysis included 6405 participants. Excluded were second members of the same household (425 participants) and participants from Site D (617 participants). When participants from Site B were also excluded, the sample size was 5859 and the adjusted hazard ratios were 0.71 (95% CI, 0.52 to 0.97) for the group assigned to a Mediterranean diet with extra-virgin olive oil, 0.68 (95% CI, 0.49 to 0.95) for the group assigned to a Mediterranean diet with nuts, and 0.69 (95% CI, 0.53 to 0.92) for the combined Mediterranean-diet groups, with the group assigned to a control diet as the reference.

0.54 to 0.95) for a Mediterranean diet with nuts (Fig. 1A and Table 3). There were similar results on three alternative analyses: one that was adjusted with inverse-probability weighting (models 3A through 3C in Fig. S2 in the Supplementary Appendix), one that included adjustments for the Framingham risk score[24] (models 6A through 6C in Fig. S2 in the Supplementary Appendix), and one that omitted participants known or suspected to have been assigned to an intervention group without individual randomization (Table 3 and Figs. 2 and 3, and Figs. S2 and S4 in the Supplementary Appendix). The results for secondary end points are shown in Table 3 and Figure 1B.

To provide an alternative, noncausal explanation of the observed association (i.e., to change the point estimate of the hazard ratio to ≥1.0), an unmeasured binary confounder would need to be present in at least 40% of the control group but in less than 25% of each Mediterranean-diet group and be associated with a relative risk of more than 4 for the primary end point. For further details, see Table S25 in the Supplementary Appendix.

To explore the effect of the October 2006 protocol change that was implemented for the control group to intensify nutritional counseling, we conducted separate analyses of the participants recruited before the protocol change and those recruited after the protocol change. The hazard ratios for the Mediterranean diet (both groups merged) as compared with the control diet were 0.77 (95% CI, 0.59 to 1.00) for the participants recruited before October 2006 and 0.49 (95% CI, 0.26 to 0.92) for those recruited in October 2006 or later (P=0.21 for heterogeneity).

The per-protocol (adherence-adjusted) hazard ratio for the primary end point was 0.42 (95% CI, 0.24 to 0.63) for the Mediterranean diet as compared with the control diet (Fig. 3); the estimated absolute differences in incidence between the combined Mediterranean-diet groups and

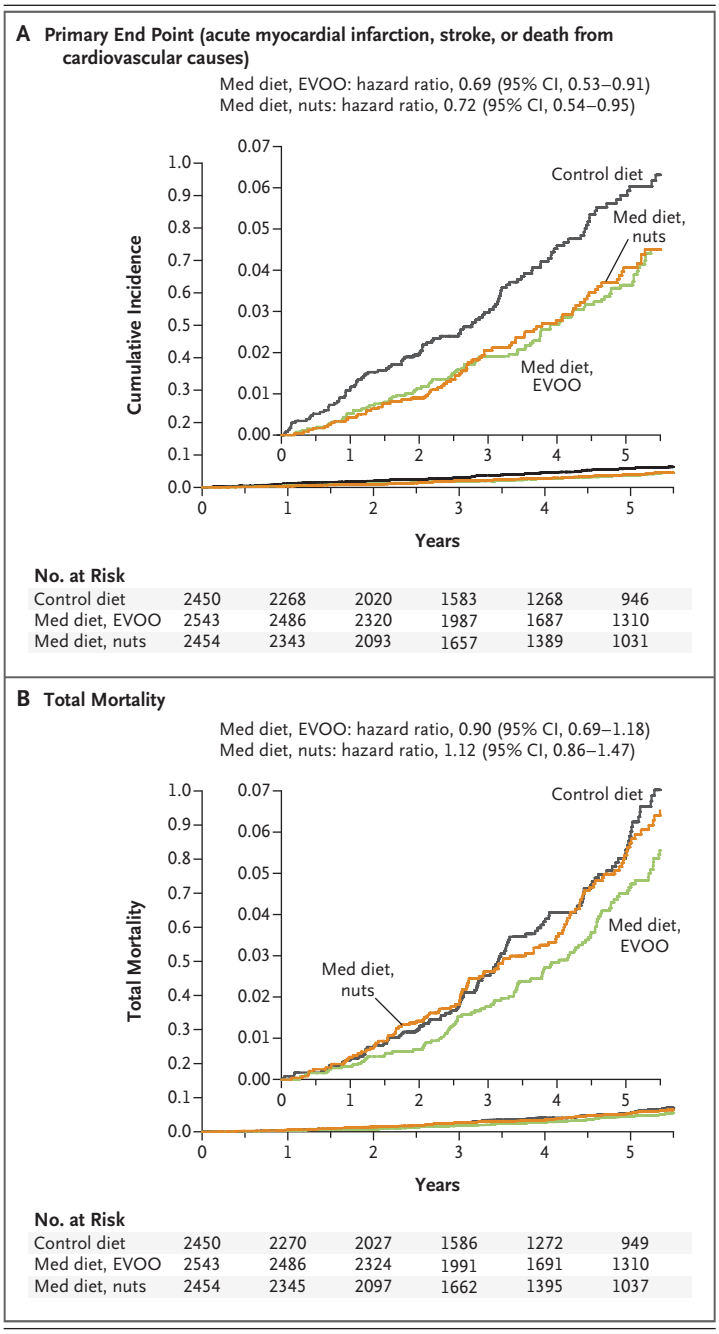

Figure 1. Kaplan–Meier Estimates of the Cumulative Incidence of End-Point Events in the Total Study Population.

Panel A shows the incidence of the primary end point (a composite of acute myocardial infarction, stroke, and death from cardiovascular causes), and Panel B shows total mortality. The insets show the same data on an expanded y axis. Hazard ratios were stratified according to sex, recruiting site, and educational level (five categories) and adjusted for age (continuous variable), smoking (never smoked, former smoker, or current smoker), hypertension (yes or no), dyslipidemia (yes or no), diabetes (yes or no), family history of premature coronary heart disease, body-mass index (continuous variable), waist-to-height ratio (continuous variable), physical activity (in quintiles), and propensity scores that estimated the probability of assignment to each intervention group on the basis of 30 baseline variables (see pages 12 through 17 in the Supplementary Appendix). Robust standard errors to account for intracluster correlations were used. CI denotes confidence interval, EVOO extra-virgin olive oil, and Med Mediterranean.

the control group were 0.67, 1.38, and 2.00 percentage points at 12, 24, and 36 months after enrollment, respectively (see pages 36 through 38 in the Supplementary Appendix). The results of additional sensitivity and subgroup analyses were also consistent with the results of our primary analyses (Figs. 2 and 3, and Figs. S2, S4, and S12 in the Supplementary Appendix).

DISCUSSION

In this study involving high-risk persons without cardiovascular disease, assignment to an energy-unrestricted Mediterranean diet supplemented with either extra-virgin olive oil or nuts was associated with a lower risk of major cardiovascular events over a period of 5 years than assignment to a control (low-fat) diet, with a relative difference of 30% and an absolute difference of 1.7 to 2.1 percentage points. Our analysis, which incorporated information about adherence to the diets, suggests that the difference in rates of cardiovascular events between those assigned to the Mediterranean diets and those assigned the control diet was greater among participants with better adherence. These results support previously reported benefits of the Mediterranean diet for cardiovascular risk reduction from a randomized trial.[4,25,26] Our findings are also consistent with those of previous observational studies.[2,5,23,25-33] Table S11 in the Supplementary Appendix summarizes the findings from systematic reviews on this issue.

In response to a 2017 report[11] suggesting that distributions of baseline variables in the PREDIMED trial were significantly different from what would have been expected to result from randomization, we conducted an extensive review

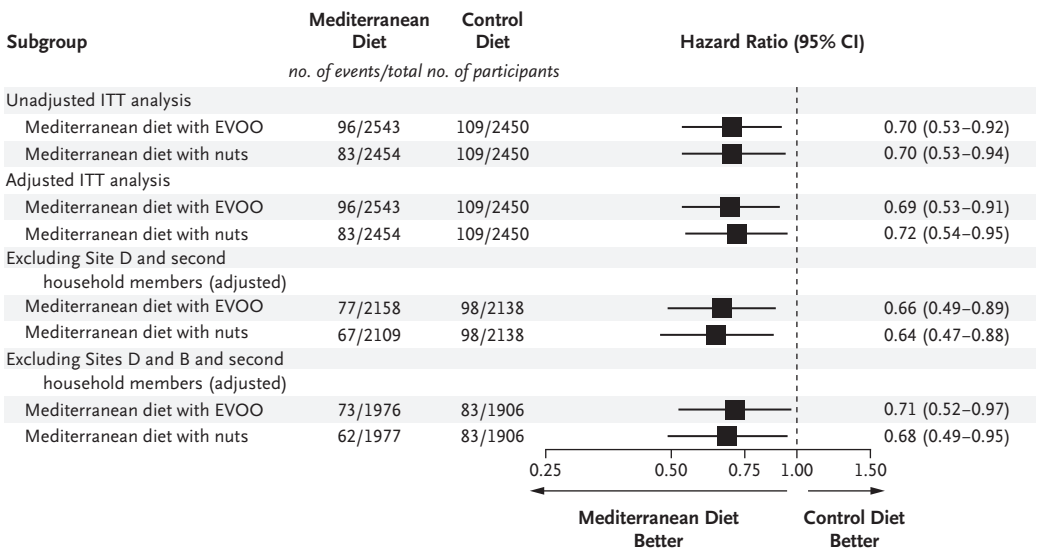

Figure 2. Sensitivity Analyses of Each Mediterranean-Diet Group and the Control Group.

Shown are hazard ratios for the primary end point in each Mediterranean-diet group as compared with the control group, with estimates based on different analytic approaches or exclusions. Hazard ratios were stratified according to sex, recruiting site, and educational level (five categories) and (with the exception of the unadjusted intention-to-treat [ITT] analysis) were adjusted for age, smoking (never smoked, former smoker, or current smoker), hypertension (yes or no), dyslipidemia (yes or no), diabetes (yes or no), family history of premature coronary heart disease, body-mass index (continuous variable), waist-to-height ratio (continuous variable), and physical activity (in quintiles). In addition, all multivariable models were adjusted for propensity scores that estimated the probability of assignment to each intervention group on the basis of 30 baseline variables. We used robust standard errors to account for intracluster correlations. One analysis excluded all the participants from Site D (652 participants) and all second members of the same household (425 participants, including 35 from Site D); in total, 1042 participants were excluded from this analysis. Another analysis excluded all the participants from Site D (652 participants), all the participants from Site B (593 participants), and all second members of the same household (425 participants, including 35 from Site D and 47 from Site B); in total, 1588 participants were excluded from this analysis. The results of additional sensitivity analyses are shown in Figures S2 and S4 in the Supplementary Appendix.

of the documentation and data at the 11 recruitment sites. After sharing this information with the editors of the *Journal*, we withdrew our original report of this trial and now publish this new report. Despite some departures from the randomization protocol, most of the baseline covariates were balanced across groups, and there was no meaningful difference in the predicted risks of future cardiovascular events across the three groups (Fig. S13 in the Supplementary Appendix).

We reanalyzed the data using methods that do not rely exclusively on the assumption that all the participants had been randomly assigned to intervention groups and that adjusted for baseline characteristics and propensity scores estimating probabilities of assignment to each intervention on the basis of 30 baseline covariates. The results of our reanalyses (Figs. 2 and 3 and Table 3, and Figs. S2 and S4 in the Supplementary Appendix) were similar to the results that we originally reported. In addition, reanalyses of our data did not reveal any evidence that certain lifestyle or treatment factors that are potentially related to the risk of cardiovascular disease either biased the results or might provide an alternative explanation for the observed benefits of the Mediterranean-diet interventions on cardiovascular disease. Analyses that excluded participants whose assignment to an intervention group was known or suspected not to have followed the randomization protocol (participants from Sites D and B and second household members) yielded results consistent with the results of our primary analysis.

The retention rate was higher in the group

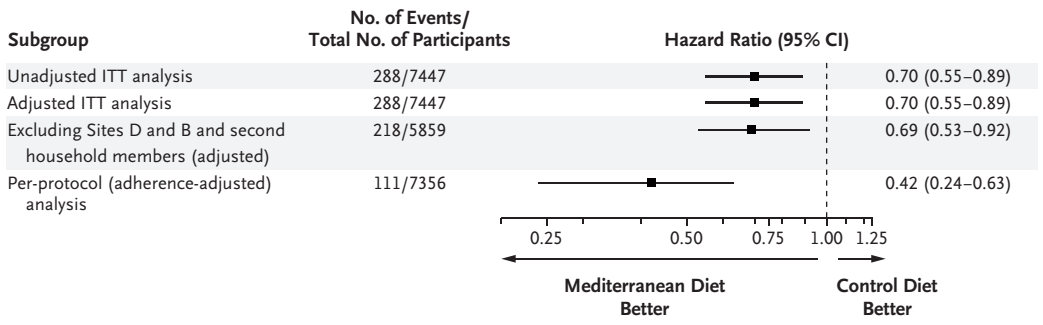

Figure 3. Sensitivity Analyses of the Combined Mediterranean-Diet Groups and the Control Group.

Shown are hazard ratios for the primary end point in the combined Mediterranean-diet groups as compared with the control group, with estimates based on different analytic approaches or exclusions. Hazard ratios were stratified according to sex, recruiting site, and educational level (five categories) and (with the exception of the unadjusted ITT analysis) were adjusted for age, smoking (never smoked, former smoker, or current smoker), hypertension (yes or no), dyslipidemia (yes or no), diabetes (yes or no), family history of premature coronary heart disease, body-mass index (continuous variable), waist-to-height ratio (continuous variable), and physical activity (in quintiles). In addition, all multivariable models were adjusted for propensity scores, and we used robust standard errors to account for intracluster correlations.

assigned to a Mediterranean diet with extra-virgin olive oil than in the other two groups (the group assigned to a Mediterranean diet with nuts and the control group). These two groups were also slightly smaller in size, which resulted in a larger number of person-years of follow-up in the group assigned to a Mediterranean diet with extra-virgin olive oil. The different follow-up had the potential to bias the incidence rates toward lower rates in the group assigned to a Mediterranean diet with extra-virgin olive oil. However, analyses that used multiple imputation and inverse-probability weighting to adjust for a potential selection bias due to differential losses to follow-up yielded estimates consistent with the main analysis (see pages 30 through 35 and Fig. S4 in the Supplementary Appendix). An additional limitation of our study is that participants were at high cardiovascular risk; whether the results can be generalized to persons at lower risk requires further research.

As with many clinical trials, the observed rates of cardiovascular events were lower than anticipated, with reduced statistical power to separately assess components of the primary end point. However, favorable trends were seen for both stroke and myocardial infarction. It is possible, but not likely, that some cardiovascular events were not detected (see pages 28 and 29 in the Supplementary Appendix).

Even though participants in the control group received advice to reduce fat intake, changes in total fat on the food-frequency questionnaire were small and the largest differences at the end of the study were in the distribution of fat subtypes. The interventions were intended to improve the overall dietary pattern, but the major between-group differences involved the supplemental items, extra-virgin olive oil and nuts. Differences were also observed in the consumption of fish and legumes but not in the consumption of other food groups. (It is worth noting that on the 14-item Mediterranean-diet questionnaire, there were substantial between-group differences in 12 of the 14 items.) The modest between-group differences according to the food-frequency questionnaire can be explained by the facts that most study participants had been consuming a baseline diet similar to the study Mediterranean diet and that the control group was given recommendations for a healthy diet, factors that raise the question of how applicable our results may be to high-risk persons in other countries. Answering this question will require further research.[3]

In conclusion, in this primary prevention study involving persons at high risk for cardiovascular events, those assigned to an energy-unrestricted Mediterranean diet, supplemented with extra-virgin olive oil or nuts, had a lower rate of major cardiovascular events than those assigned to a

reduced-fat diet. Our findings support a beneficial effect of the Mediterranean diet for the primary prevention of cardiovascular disease.

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APPENDIX

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ORIGINAL RESEARCH

Dietary Patterns and Mediterranean Diet Score and Hazard of Recurrent Coronary Heart Disease Events and All-Cause Mortality in the REGARDS Study

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***Background*—**Previously, we reported on associations between dietary patterns and incident acute coronary heart disease (CHD) in the REGARDS (Reasons for Geographic and Racial Differences in Stroke) study. Here, we investigated the associations of dietary patterns and a dietary index with recurrent CHD events and all-cause mortality in REGARDS participants with existing CHD.

***Methods and Results*—**We included data from 3562 participants with existing CHD in REGARDS. We used Cox proportional hazards regression to examine the hazard of first recurrence of CHD events—definite or probable MI or acute CHD death—and all-cause mortality associated with quartiles of empirically derived dietary patterns (convenience, plant-based, sweets, Southern, and alcohol and salads) and the Mediterranean diet score. Over a median 7.1 years (interquartile range, 4.4, 8.9 years) follow-up, there were 581 recurrent CHD events and 1098 deaths. In multivariable-adjusted models, the Mediterranean diet score was inversely associated with the hazard of recurrent CHD events (hazard ratio for highest score versus lowest score, 0.78; 95% confidence interval, 0.62–0.98; P_{Trend}=0.036). The Southern dietary pattern was adversely associated with the hazard of all-cause mortality (hazard ratio for Q4 versus Q1, 1.57; 95% confidence interval, 1.28–1.91; P_{Trend}<0.001). The Mediterranean diet score was inversely associated with the hazard of all-cause mortality (hazard ratio for highest score versus lowest score, 0.80; 95% confidence interval, 0.67–0.95; P_{Trend}=0.014).

***Conclusions*—**The Southern dietary pattern was associated with a greater hazard of all-cause mortality in REGARDS participants. Greater adherence to the Mediterranean diet was associated with both a lower hazard of recurrent CHD events and all-cause mortality. **(*J Am Heart Assoc*. 2018;7:e008078. DOI: 10.1161/JAHA.117.008078.)**

Key Words: cardiovascular disease prevention • diet • epidemiology • nutrition

Despite decades of research and improvements in treatment, coronary heart disease (CHD) remains an important cause of death in the United States, accounting for 1 of every 7 deaths in 2014—more than 364 000 deaths in total.[1] Risk factors for CHD are well established and include dyslipidemia, diabetes mellitus, hypertension, overweight/obesity, cigarette smoking, and physical inactivity.[2] Observational and intervention studies provide evidence that diet also influences risk of CHD, as well as the course of the disease, likely through its documented effects on several of these key risk factors.[3]

Studies of diet and CHD risk traditionally focus on dietary constituents such as individual foods and nutrients, resulting in important findings such as the adverse associations of red meat and saturated fat with CHD risk.[4] However, interest in overall diet and CHD risk has increased in the past decade with the understanding—by both researchers and the public—that foods typically are eaten in combination, not in isolation.[5] Therefore, a comprehensive dietary approach more closely reflects the way most humans actually eat.

Empirically deriving dietary patterns a posteriori has facilitated investigations into the role overall diet may play

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Clinical Perspective

What Is New?

- Greater adherence to a "Southern" dietary pattern, characterized by added fats, fried food, eggs and egg dishes, organ meats, processed meats, and sugar-sweetened beverages, was associated with a greater hazard of all-cause mortality in community-dwelling blacks and whites who were at least 45 years of age.
- Greater adherence to the Southern dietary pattern was not associated with hazard of recurrent coronary heart disease events.
- A higher Mediterranean diet score (indicating greater adherence to the Mediterranean diet) was associated with a lower hazard of both recurrent coronary heart disease events and all-cause mortality.
- Although these are observational data, this study was conducted in a large, population-based, and diverse sample and included a comprehensive assessment of diet and a rigorous method for deriving dietary patterns.

What Are the Clinical Implications?

- Based on these results, it would be reasonable to make recommendations to patients to reduce intakes of the main components of the Southern dietary pattern, and more closely adhere to the principles of the Mediterranean diet.
- There are no known risks associated with these recommendations, and they may favorably influence the hazard of recurrent coronary heart disease and all-cause mortality.

in the etiology of chronic diseases.[6,7] Factor analysis, a data-driven exploratory method, assesses eating patterns in specified groups without preconceived judgments about which foods commonly are consumed together and has been used in previous studies to derive dietary patterns that subsequently were related to CHD risk.[8–16] In a previous analysis, we derived dietary patterns with factor analysis within the REGARDS (Reasons for Geographic and Racial Differences in Stroke) study, a national, population-based, longitudinal cohort.[17] We then used Cox proportional hazards regression to examine hazard of incident acute CHD events—nonfatal myocardial infarction and acute CHD death—associated with quartiles of consumption of each pattern in participants free of CHD at baseline.[18] After multivariable adjustment, the highest consumers of the Southern pattern (characterized by added fats, fried food, eggs, organ and processed meats, and sugar-sweetened beverages) experienced a 56% higher hazard of incident acute CHD.

While examining risk factors for incident CHD is critical for primary prevention, individuals who suffer a myocardial infarction (MI) now have greater survival than in previous years.[19] As a result, there is growing interest in examining risk factors for recurrent events among those with established disease. Therefore, we investigated the association of REGARDS dietary patterns, along with a dietary index—the Mediterranean diet score—with *recurrent* CHD events and all-cause mortality in REGARDS participants with CHD at baseline.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

Details on the design and methods of REGARDS have been published.[20] Briefly, REGARDS is a national, population-based, longitudinal cohort of 30 239 community-dwelling black and white women and men ≥45 years of age, identified via mail and telephone using commercially available lists of US residents, and enrolled from 2003 to 2007. The sampling scheme included 30% of participants from the stroke belt (North Carolina, South Carolina, Georgia, Tennessee, Alabama, Mississippi, Arkansas, and Louisiana), 20% from the stroke buckle (the coastal plain of North Carolina, South Carolina, and Georgia), and 50% from elsewhere in the continental United States. The baseline cohort was 42% black and 55% women.

Exclusion criteria included race other than white or black, active treatment for cancer, chronic medical conditions precluding long-term participation, cognitive impairment, current or impending residence in a nursing home, or inability to communicate in English. An initial telephone interview was used to survey participants and establish eligibility. Following verbal consent, demographic information and medical history (including risk factor evaluation) was collected by computer-assisted telephone interviewing. Race was self-classified by participants and included the following options defined by the investigators: white and black/African American. An in-home examination was conducted to perform various physical measurements, medication inventory, ECG, phlebotomy, and urine collection among those eligible. The study was approved by the institutional review boards at all participating institutions, and written informed consent was obtained from all participants.

For this analysis, we included only those REGARDS participants with a history of CHD at baseline (n=5314), defined as self-reported history of MI or coronary revascularization procedure, or evidence of MI on the baseline ECG.

Dietary Assessment

Diet was assessed with the Block 98 food frequency questionnaire (FFQ), a validated semiquantitative FFQ that assessed usual dietary intake of 110 food items (NutritionQuest, Berkeley, CA).[21,22] For each line item on the FFQ,

participants were asked how often, on average, they consumed the food (or group of foods) during the previous year, as well as the quantity of the food consumed. The FFQ included adjustment questions (eg, inquiring about the type of milk consumed—low-fat, nonfat, etc). The FFQ was self-administered after the in-home visit and mailed to the REGARDS Operations Center, where they were checked for completeness, scanned, and forwarded to NutritionQuest for processing and analysis. Amounts of each food on the FFQ consumed by a participant were calculated by multiplying the frequency of consumption of that food by the usual amount consumed. Calculation of the total weight (g) of each line item on the FFQ was provided by NutritionQuest. A total of 56 food groups, on which dietary patterns were based, were derived using the 110 individual food variables on the FFQ using methods described elsewhere.[17]

Dietary Patterns

We used split sample replication to (1) derive the dietary patterns using exploratory factor analysis and (2) test the patterns using confirmatory factor analysis.[23] We conducted 3 separate analyses: by sex (male/female), race (black/white), and region (southeastern US stroke belt/nonbelt), and coefficients of congruence were determined for each stratification pair. The final number of factors retained was chosen based on the eigenvalue (scree plot) and the solution providing the optimal congruence across sex, race, and region. As congruence between sex, race, and region was high, we calculated final factor loadings using factor analysis with varimax rotation of 5 factors on the full sample. We named patterns based on the factor loadings that contributed most highly to each pattern. Factor 1 loaded heavily on mixed dishes, pasta dishes, pizza, Mexican food, and Chinese food and was designated the "convenience" pattern. Factor 2 had high factor loadings for vegetables, fruits, fruit juice, cereal, beans, fish, poultry, and yogurt and was named the "plant-based" pattern. Factor 3 loaded on added sugars, desserts, chocolate, candy, and sweetened breakfast foods and was named the "sweets" pattern. Factor 4 loaded heavily on added fats, fried food, eggs and egg dishes, organ meats, processed meats, and sugar-sweetened beverages. This diet reflected a culinary pattern observed in the southeastern US and was named the "Southern" pattern. Factor 5 loaded highly on beer, wine, liquor, green leafy vegetables, tomatoes, and salad dressing. Accordingly, we named it the "alcohol and salads" pattern.

Mediterranean Diet Score

We included the Mediterranean diet score because it has been associated with reduced risk of chronic disease incidence and mortality in various populations.[24] The Mediterranean diet score was derived according to previously published methods used in REGARDS.[25] In brief, food group contributors to the Mediterranean diet score included those designated as "beneficial" (vegetables, fruits, legumes, cereals, fish), and those designated as "detrimental" (meat, dairy). One point was assigned for consumption that exceeded the median for the "beneficial" groups or was below the median for "detrimental" food groups. For fat intake (eighth food category) we used the ratio of daily consumption (in grams) of monounsaturated lipids to saturated lipids, and we calculated the median separately for each sex. Individuals with ratios at or above the sex-specific median were assigned a value of 1, and those with ratios below the sex-specific median were assigned a value of 0. Moderate alcohol (ninth food category) consumption was defined as >0 and ≤ 7 drinks per week for women and >0 and ≤ 14 drinks per week for men. More-than-moderate consumption was defined as >7 drinks per week for women and >14 drinks per week for men. Individuals were assigned a score of 1 for moderate consumption and a score of 0 for the other 2 categories (0 and more-than-moderate consumption). Summing scores for the 9 food groups resulted in a possible score of 0 to 9, with a higher score reflecting higher adherence to the Mediterranean diet.

Outcome Ascertainment

We defined recurrent CHD events as first occurrence of definite or probable MI or acute CHD death in participants with a history (defined above) of CHD at baseline. Recurrent cases of CHD were captured by participant report and adjudicated by clinicians with appropriate expertise. Participants were contacted by telephone every 6 months to assess vital status. If a suspected heart event was reported, medical records were pursued. MIs were adjudicated based on the presence of signs or symptoms suggestive of ischemia; diagnostic cardiac enzymes (rising and/or falling pattern in cardiac troponin or creatine phosphokinase-MB isoenzyme concentrations over ≥ 6 hours with a peak concentration greater than twice the upper limit of normal); and ECG changes consistent with ischemia or MI, guided by the Minnesota Code and classified as evolving diagnostic, positive, nonspecific, or not consistent with ischemia.[26] In the case of deaths, interviews with family members or other proxies, proximal hospitalizations, baseline medical history, death certificates, and the National Death Index were used to identify CHD as the underlying cause of death for analyses of recurrent CHD events or to identify any death for analyses of all-cause mortality.

Statistical Analysis

Of the participants with a history of CHD at baseline (n=5314), we excluded 1671 participants who were missing FFQ data altogether, had >15% missing data on the FFQ, or

had implausible reported energy intakes (<800 or >5000 kcal/day in men and <500 or >4500 kcal/day in women), precluding derivation of dietary patterns and/or Mediterranean diet score. In addition, we excluded 81 participants who were lost to follow-up. This resulted in a final sample of 3562 (67.0% of the sample with a history of CHD at baseline) (see Figure).

We categorized consumption of the 5 dietary patterns into quartiles, with quartile 1 representing the lowest consumption of each pattern and quartile 4 representing the highest consumption of the pattern. We categorized the Mediterranean diet score into 3 groups representing lowest to highest consumption of the Mediterranean diet. We calculated descriptive statistics (including proportions and measures of central tendency) for demographic, socioeconomic, lifestyle, anthropometric, medical history, and medication variables at the baseline assessment according to these quartiles/groups using the chi-square test (for proportions) and ANOVA (for continuous variables). We used Cox proportional hazards regression to examine the hazard of recurrent CHD events and all-cause mortality associated with consumption of each of the 5 dietary patterns and Mediterranean diet score, using the lowest quartile/group of consumption (quartile/group 1) as the referent quartile/group throughout. Quartiles proved not to be appropriate for analyses of the Mediterranean diet score. Specifically, attempting to categorize Mediterranean diet scores (originally on a scale of 0–9 to assess adherence) into 4 somewhat uniform groups was not possible because of the distribution of the scores—this would have resulted in at least 1 category with a very small number compared with the other groups. Therefore, 3 more equal groups were created based on the scores 0 to 3, 4 to 5, and 6 to 9. Years since study entry was the time metric, with participants censored at the date of a recurrent CHD event (for the recurrent CHD

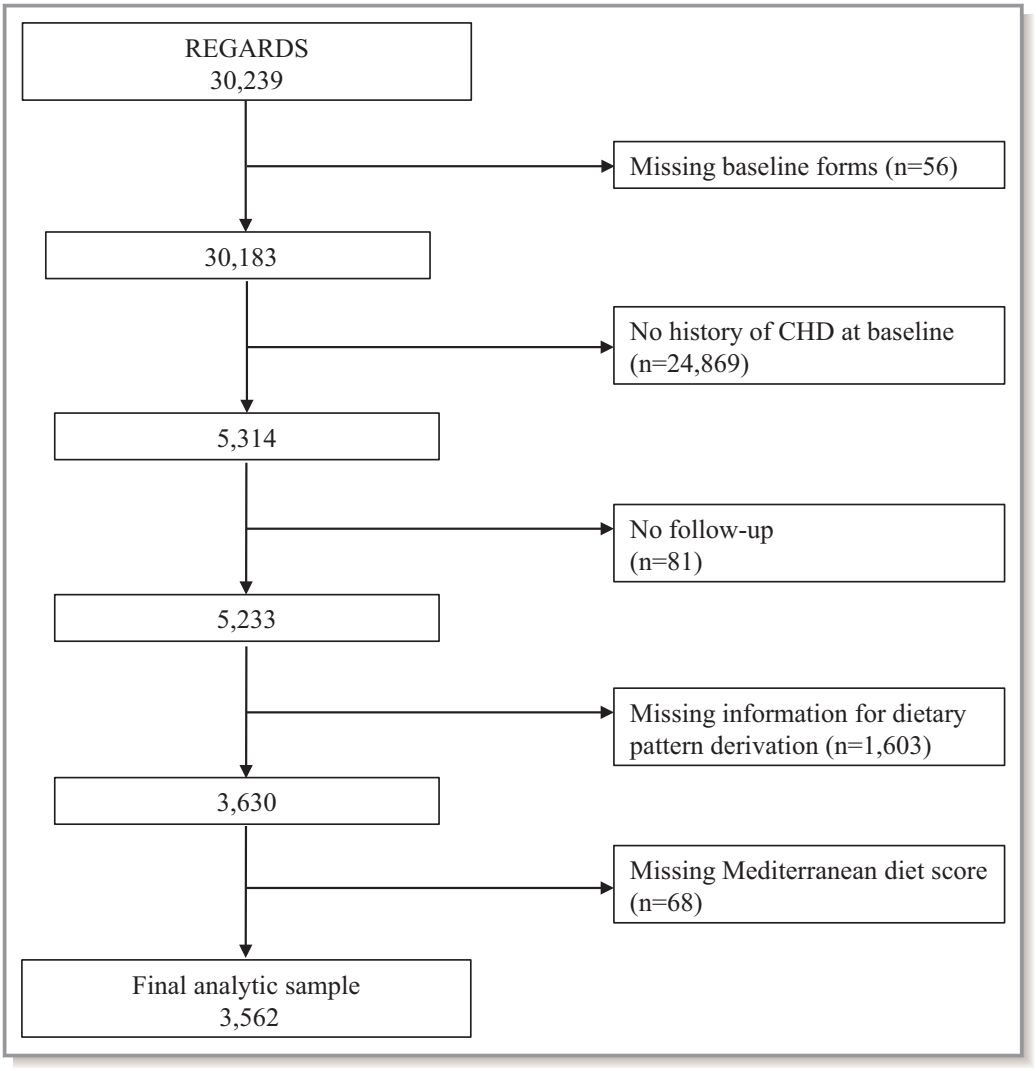

Figure. Participant exclusion cascade. CHD indicates coronary heart disease; REGARDS, Reasons for Geographic and Racial Differences in Stroke.

analysis); date of withdrawal from the study; date of death; or December 31, 2013, whichever came first. We examined Schoenfeld residuals and confirmed that proportional hazards assumptions were met. The base model (model 1) included the demographic variables age, sex, and race. The multivariable-adjusted model (model 2) included factors in model 1 plus socioeconomic factors (education, household income), region, lifestyle factors (smoking, physical activity), total energy intake, anthropometric factors (body mass index [BMI], waist circumference), systolic blood pressure, medical history (hypertension, dyslipidemia, diabetes mellitus), and a physical health summary scale: the Physical Component Summary from the 12-item Short-Form Health Survey.

A Wald test was conducted to assess possible effect modification by sex and race. We conducted a further sensitivity analysis including antihypertensive, antidyslipidemic, and antidiabetic medications in model 2. To address concerns of collinearity between waist circumference and BMI, we ran all models using residualized variables. In a final sensitivity analysis, we used multiple imputation by chained equations to impute both the main exposure and covariates.

A total of 971 participants (27.3% of the 3562 participants) were missing information for at least 1 covariate of interest. Variables with the largest amount of missing data included income, antihypertensive medication and insulin use, and physical health summary scale. All other characteristics had ≤1% missing data. We performed analyses using SAS statistical software, version 9.4 (SAS Institute, Cary, NC) and Stata, version 14.2 (StataCorp, College Station, TX). A P value of <0.05 was considered statistically significant. One author (M.M.S.) had full access to all the data in the study and takes responsibility for its integrity and the data analysis.

Results

Over a median 7.1 years follow-up (interquartile range, 4.4, 8.9 years), there were 581 (16.3%) recurrent CHD events and 1098 (30.8%) deaths. Compared with participants without recurrent CHD events, those with recurrent CHD events were older (70.0±9.2 versus 68.3±8.8 years), more likely to be male (70.1% versus 59.4%), more likely to not have graduated from high school (16.2% versus 12.9%), and more likely to be physically inactive (44.9% versus 34.6%) (Table 1). Participants with recurrent CHD events also had higher BMI (29.9±5.7 versus 29.0±5.9 kg/m^2), waist circumference (102.2±15.3 versus 98.3±15.4 cm), and systolic blood pressure (132.3±17.9 versus 128.6±16.7 mm Hg); were more likely to have a history of hypertension (77.6% versus 69.3%), dyslipidemia (81.9% versus 77.2%), and diabetes mellitus (42.7% versus 27.3%); were more likely to report antihypertensive medication (72.2% versus 65.9%), regular aspirin (75.0% versus 69.7%), oral antidiabetic medication (30.5% versus 19.5%), and insulin (16.3% versus 8.7%) use; and had a lower physical health summary score (40.2±11.4 versus 43.5±11.4).

In multivariable-adjusted models, the plant-based dietary pattern demonstrated a trend for an increasing hazard of recurrent CHD events, although none of the hazard ratios (HRs) for quartile comparisons were statistically significant (HR for Q4 versus Q1, 1.28; 95% confidence interval [CI], 0.98–1.66; P_{Trend}=0.048) (Table 2). The Southern pattern was adversely associated with the hazard of recurrent CHD events in the minimally adjusted model (HR for Q4 versus Q1, 1.35; 95% CI, 1.05–1.73; P_{Trend}=0.011). However, the Southern pattern was not associated with the hazard of recurrent CHD events in the fully adjusted model (HR for Q4 versus Q1, 1.00; 95% CI, 0.76–1.31; P_{Trend}=0.942). After multivariable adjustment, the alcohol and salads pattern was inversely associated with the hazard of recurrent CHD events (HR for Q4 versus Q1, 0.77; 95% CI, 0.59–1.00; P_{Trend}=0.026). None of the other dietary patterns were significantly associated with hazard of recurrent CHD events. The multivariable-adjusted Mediterranean diet score was inversely associated with the hazard of recurrent CHD events (HR for group 3 versus group 1, 0.78; 95% CI, 0.62–0.98; P_{Trend}=0.036) (Table 3).

In multivariable-adjusted all-cause mortality analyses, the Southern dietary pattern was adversely associated with the hazard of all-cause mortality (HR for Q4 versus Q1, 1.57; 95% CI, 1.28–1.91; P_{Trend}<0.001) (Table 4). The plant-based pattern was inversely associated with the hazard of all-cause mortality in the minimally adjusted model (HR for Q4 versus Q1, 0.71; 95% CI, 0.59–0.84; P_{Trend}<0.001). While this association was attenuated in the fully adjusted model, evidence of an inverse association of the plant-based pattern with the hazard of all-cause mortality remained (HR for Q4 versus Q1, 0.84; 95% CI, 0.69–1.01; P_{Trend}=0.150). None of the other empirically derived dietary patterns were associated with the hazard of all-cause mortality. The multivariable-adjusted Mediterranean diet score was inversely associated with the hazard of all-cause mortality (HR for group 3 versus group 1, 0.80; 95% CI, 0.67–0.95; P_{Trend}=0.014) (Table 5).

Sensitivity analyses solidified the results above. There was no statistically significant interaction by race or sex. Additionally, estimates were virtually unchanged when antihypertensive, antidyslipidemic, and antidiabetic medications were added to the final model; waist circumference and BMI were residualized; or the main exposure and covariates were imputed.

Discussion

In this follow-up to our analysis of dietary patterns and incident acute CHD in the REGARDS cohort, a Southern dietary pattern (characterized by added fats, fried food, eggs, organ meats, processed meats, and sugar-sweetened

Table 1. Characteristics of Study Participants Without and With Recurrent CHD Events in the REGARDS Cohort

Characteristic	Total Mean±SD or n (%) N=3562	No Recurrent CHD Event Mean±SD or n (%) n=2981	Recurrent CHD Event Mean±SD or n (%) n=581	$P_{Difference}$
Age, y	68.6±8.9	68.3±8.8	70.0±9.2	<0.001
Sex, male	2179 (61.2)	1772 (59.4)	407 (70.1)	<0.001
Race, black	962 (27.0)	812 (27.2)	150 (25.8)	0.48
Education, did not graduate from high school	477 (13.4)	383 (12.9)	94 (16.2)	0.031
Household income <$20 000/y	686 (21.7)	557 (21.1)	129 (24.4)	0.092
Resident of stroke belt*	2007 (56.3)	1692 (56.8)	315 (54.2)	0.26
Current smoker	527 (14.9)	434 (14.6)	93 (16.0)	0.38
Physically inactive†	1276 (36.3)	1020 (34.6)	256 (44.9)	<0.001
Total energy intake, kcal/day	1685±688	1684±687	1694±693	0.74
Body mass index, kg/m^2	29.2±5.8	29.0±5.9	29.9±5.7	0.002
Waist circumference, cm	98.9±15.4	98.3±15.4	102.2±15.3	<0.001
Systolic blood pressure, mm Hg	129.2±16.9	128.6±16.7	132.3±17.9	<0.001
Hypertension‡	2506 (70.7)	2058 (69.3)	448 (77.6)	<0.001
Dyslipidemia§	2716 (78.0)	2251 (77.2)	465 (81.9)	0.015
Diabetes mellitus‖	1030 (29.8)	786 (27.3)	244 (42.7)	<0.001
Antihypertensive use	2298 (66.9)	1887 (65.9)	411 (72.2)	0.003
Statin use	2109 (59.2)	1759 (59.0)	350 (60.2)	0.58
Regular aspirin use	2512 (70.5)	2077 (69.7)	435 (75.0)	0.010
Oral antidiabetic use	759 (21.3)	582 (19.5)	177 (30.5)	<0.001
Insulin use	334 (9.9)	245 (8.7)	89 (16.3)	<0.001
PCS-12	43.0±11.5	43.5±11.4	40.2±11.4	<0.001

CHD indicates coronary heart disease; PCS, Physical Component Summary; REGARDS, Reasons for Geographic and Racial Differences in Stroke study; SD, standard deviation.
*Stroke belt consists of North Carolina, South Carolina, Georgia, Tennessee, Alabama, Mississippi, Arkansas, and Louisiana.
†Physically active defined as ≥4 days of exercise (enough to work up a sweat) per week.
‡Hypertension defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or self-reported current use of medication to control blood pressure.
§Dyslipidemia defined as total cholesterol ≥6.22 mmol/L (240 mg/dL) and/or low-density lipoprotein cholesterol ≥4.14 mmol/L (160 mg/dL) and/or high-density lipoprotein cholesterol ≤1.04 mmol/L (40 mg/dL) or self-reported current use of medication to control cholesterol.
‖Diabetes mellitus defined as fasting glucose ≥6.99 mmol/L (126 mg/dL) and/or nonfasting glucose ≥11.10 mmol/L (200 mg/dL) or self-reported current use of medication to control blood sugar.

beverages) was not associated with the hazard of recurrent CHD events, in contrast to the adverse association of this dietary pattern with the hazard of incident acute CHD.[18] The Southern pattern was adversely associated with the hazard of all-cause mortality in REGARDS participants with existing CHD. Interestingly, greater adherence to the plant-based dietary pattern showed a significant trend of a greater hazard of recurrent CHD events, although none of the HRs for quartile comparisons were statistically significant. Higher consumption of a Mediterranean diet was inversely associated with both hazard of recurrent CHD events and all-cause mortality.

Although previous studies have investigated the association of dietary patterns with incident CHD events, CHD risk factors, post-MI prognosis and cardiovascular mortality, and coronary procedures such as angioplasty or coronary artery bypass graft surgery, ours is among the first to investigate a posteriori–derived dietary patterns and hazard of recurrent CHD events using adjudication, a more rigorous assessment than self-report. This is in contrast to the Mediterranean diet, where clinical trial data from the Lyon Diet Heart Study have shown that patients with a previous MI who were randomized to consume a Mediterranean diet had a lower rate of recurrent MI compared with patients randomized to a prudent-type diet.[27] However, because of the relatively small sample size and short duration of follow-up in the Lyon Study, as well as the selective nature of recruitment in this and other clinical trials, observational data from cohorts of community-dwelling people, especially those such as REGARDS, which include a significant proportion of black participants, remain relevant. A

Table 2. Hazard of Recurrent CHD Events by Quartile of Consumption of the Various Dietary Patterns

Dietary Pattern	Model	Quartile 1 (Lowest Consumption), HR (95% CI)	Quartile 2, HR (95% CI)	Quartile 3, HR (95% CI)	Quartile 4 (Highest Consumption), HR (95% CI)	P_{Trend}
Convenience		n=894 (158*)	n=890 (137)	n=888 (138)	n=890 (148)	
		279.4†	237.2	235.8	246.0	
	1‡	1 (referent)	0.87 (0.69–1.09)	0.85 (0.68–1.08)	0.92 (0.73–1.16)	0.472
	2§	1 (referent)	0.83 (0.66–1.05)	0.86 (0.67–1.09)	0.87 (0.67–1.13)	0.296
Plant-based		n=887 (135)	n=894 (146)	n=891 (155)	n=890 (145)	
		238.9	251.0	261.5	245.6	
	1	1 (referent)	0.99 (0.78–1.25)	1.02 (0.81–1.29)	0.97 (0.76–1.23)	0.863
	2	1 (referent)	1.08 (0.85–1.37)	1.19 (0.93–1.51)	1.28 (0.98–1.66)	0.048
Sweets		n=892 (132)	n=888 (152)	n=890 (149)	n=892 (148)	
		226.4	262.9	249.9	258.5	
	1	1 (referent)	1.11 (0.88–1.41)	1.05 (0.83–1.33)	1.10 (0.87–1.39)	0.590
	2	1 (referent)	1.05 (0.83–1.33)	1.00 (0.78–1.28)	1.11 (0.83–1.48)	0.640
Southern		n=895 (134)	n=888 (138)	n=891 (154)	n=888 (155)	
		215.5	236.7	267.9	281.6	
	1	1 (referent)	1.12 (0.88–1.42)	1.26 (1.00–1.60)	1.35 (1.05–1.73)	0.011
	2	1 (referent)	0.99 (0.78–1.27)	1.02 (0.80–1.30)	1.00 (0.76–1.31)	0.942
Alcohol and salads		n=886 (149)	n=887 (158)	n=895 (148)	n=894 (126)	
		263.6	281.0	252.1	204.7	
	1	1 (referent)	1.07 (0.85–1.34)	0.95 (0.75–1.20)	0.75 (0.59–0.96)	0.015
	2	1 (referent)	1.11 (0.89–1.39)	0.93 (0.73–1.17)	0.77 (0.59–1.00)	0.026

CHD indicates coronary heart disease; CI, confidence interval; HR, hazard ratio; PCS, Physical Component Summary.
*Number of events.
†Crude rate of recurrent coronary heart disease events per 10 000 person-years.
‡Model 1 adjusts for age, sex, and race.
§Model 2 adjusts for age, sex, race, education, household income, region, smoking, physical activity, total energy intake, body mass index, waist circumference, systolic blood pressure, history of hypertension, dyslipidemia, diabetes mellitus, and PCS-12.

previous analysis investigating various lifestyle modifications and recurrent CHD events in REGARDS showed an inverse association between the Mediterranean diet score and risk of recurrent CHD events, although this finding was of borderline statistical significant (HR [95% CI] for Q4 versus Q1, 0.77 [0.55–1.06]; P_{Trend}=0.084).[28] However, that analysis included outcome data through December 31, 2009, while the current analysis includes outcomes occurring over 4 additional years (through December 31, 2013).

Previous studies of dietary patterns and all-cause mortality in various populations have produced mixed results. Western-type diets (high-fat, meat-rich, low-fiber), which have similarities to our Southern dietary pattern, have shown adverse associations with all-cause mortality in several cohorts, including the US Nurse's Health study,[29] older British men,[30] and Chinese men and women (but only in ever smokers).[31] However, a Western dietary pattern showed no association with all-cause mortality in a Spanish cohort,[32] in English civil service employees in the Whitehall II study,[33] or in a cohort of Danish men and women.[34] There was a surprising *inverse* association of a Western dietary pattern and all-cause mortality in a cohort of Japanese men and women.[35] In a recent systematic review and meta-analysis of 13 prospective cohort studies, a Western dietary pattern was not significantly associated with risk of all-cause mortality.[36]

In contrast to the lack of an association with the plant-based pattern and all-cause mortality in our analysis, a prudent diet (which generally is characterized by high intakes of fruits and vegetables, and low loadings of meats and sweets) was associated with a significantly lower risk of all-cause mortality in the Nurse's Health Study cohort,[29] a Chinese population,[31] Danish men and women,[34] and a cohort of Japanese men and women.[35] However, there was no association of healthy dietary patterns with all-cause mortality in a study of British men,[30] or in the Whitehall II study.[33] In the previously noted systematic review and meta-analysis, a prudent dietary pattern was inversely associated with risk of all-cause mortality.[36]

Table 3. Hazard of Recurrent CHD Events by Mediterranean Diet Score Group

Diet Score	Model	Group 1 (Score 0–3), HR (95% CI)	Group 2 (Score 4, 5), HR (95% CI)	Group 3 (Score 6–9), HR (95% CI)	P_{Trend}
Mediterranean		n=1145 (208*)	n=1500 (248)	n=917 (125)	
		291.2†	252.4	197.4	
	1‡	1 (referent)	0.81 (0.67–0.97)	0.61 (0.49–0.76)	<0.001
	2§	1 (referent)	0.91 (0.76–1.10)	0.78 (0.62–0.98)	0.036

CHD indicates coronary heart disease; CI, confidence interval; HR, hazard ratio; PCS, Physical Component Summary.
*Number of events.
†Crude rate of recurrent coronary heart disease events per 10 000 person-years.
‡Model 1 adjusts for age, sex, and race.
§Model 2 adjusts for age, sex, race, education, household income, region, smoking, physical activity, total energy intake, body mass index, waist circumference, systolic blood pressure, history of hypertension, dyslipidemia, diabetes mellitus, and PCS-12.

In agreement with our results, a Mediterranean dietary pattern was inversely associated with all-cause mortality in the US Multiethnic Cohort,[37] the US National Institutes of Health–AARP Diet and Health Study,[38] Spanish cohorts,[32,39] an Italian cohort,[40] the UK-based EPIC (European Prospective Investigation of Cancer)–Norfolk study,[41] a Danish cohort,[42] and a cohort of elderly European men and women.[43] However, there was no association of a Mediterranean-type diet with all-cause mortality in the UK-based Whitehall II study.[33] A previous analysis investigating various lifestyle modifications

Table 4. Hazard of All-Cause Mortality by Quartile of Consumption of the Various Dietary Patterns

Dietary Pattern	Model	Quartile 1 (Lowest Consumption), HR (95% CI)	Quartile 2, HR (95% CI)	Quartile 3, HR (95% CI)	Quartile 4 (Highest Consumption), HR (95% CI)	P_{Trend}
Convenience		n=894 (306*)	n=890 (280)	n=888 (261)	n=890 (251)	
		513.3†	461.2	425.6	398.6	
	1‡	1 (referent)	0.95 (0.81–1.12)	0.92 (0.77–1.09)	0.96 (0.80–1.14)	0.527
	2§	1 (referent)	0.92 (0.78–1.09)	0.97 (0.81–1.15)	0.89 (0.74–1.08)	0.342
Plant-based		n=887 (269)	n=894 (282)	n=891 (294)	n=890 (253)	
		454.8	459.5	476.0	405.9	
	1	1 (referent)	0.85 (0.71–1.00)	0.86 (0.73–1.01)	0.71 (0.59–0.84)	<0.001
	2	1 (referent)	0.90 (0.76–1.07)	0.96 (0.81–1.15)	0.84 (0.69–1.01)	0.150
Sweets		n=892 (258)	n=888 (284)	n=890 (275)	n=892 (281)	
		421.2	469.4	440.0	465.6	
	1	1 (referent)	1.00 (0.85–1.19)	0.94 (0.80–1.12)	1.05 (0.88–1.24)	0.781
	2	1 (referent)	0.94 (0.79–1.12)	0.87 (0.72–1.04)	0.94 (0.76–1.16)	0.358
Southern		n=895 (216)	n=888 (262)	n=891 (300)	n=888 (320)	
		330.7	428.3	496.2	554.8	
	1	1 (referent)	1.34 (1.12–1.61)	1.61 (1.35–1.93)	2.01 (1.67–2.41)	<0.001
	2	1 (referent)	1.21 (1.01–1.46)	1.37 (1.15–1.65)	1.57 (1.28–1.91)	<0.001
Alcohol and salads		n=886 (292)	n=887 (291)	n=895 (279)	n=894 (236)	
		489.7	487.0	452.9	370.9	
	1	1 (referent)	1.03 (0.88–1.22)	1.01 (0.85–1.19)	0.82 (0.69–0.98)	0.032
	2	1 (referent)	1.09 (0.92–1.29)	1.03 (0.86–1.22)	0.86 (0.71–1.03)	0.117

CI indicates confidence interval; HR, hazard ratio; PCS, Physical Component Summary.
*Number of events.
†Crude rate of all-cause mortality per 10 000 person-years.
‡Model 1 adjusts for age, sex, and race.
§Model 2 adjusts for age, sex, race, education, household income, region, smoking, physical activity, total energy intake, body mass index, waist circumference, systolic blood pressure, history of hypertension, dyslipidemia, diabetes mellitus, and PCS-12.

Table 5. Hazard of All-Cause Mortality by Mediterranean Diet Score Group

Diet Score	Model	Group 1 (Score 0–3), HR (95% CI)	Group 2 (Score 4, 5), HR (95% CI)	Group 3 (Score 6–9), HR (95% CI)	P_{Trend}
Mediterranean		n=1145 (379*)	n=1500 (481)	n=917 (238)	
		499.9†	468.7	359.6	
	1‡	1 (referent)	0.84 (0.73–0.96)	0.60 (0.51–0.71)	<0.001
	2§	1 (referent)	0.98 (0.85–1.13)	0.80 (0.67–0.95)	0.014

CI indicates confidence interval; HR, hazard ratio; PCS, Physical Component Summary.
*Number of events.
†Crude rate of all-cause mortality per 10 000 person-years.
‡Model 1 adjusts for age, sex, and race.
§Model 2 adjusts for age, sex, race, education, household income, region, smoking, physical activity, total energy intake, body mass index, waist circumference, systolic blood pressure, history of hypertension, dyslipidemia, diabetes mellitus, and PCS-12.

and recurrent CHD events in REGARDS showed an inverse association between the Mediterranean diet score and risk of all-cause mortality, although this finding was of borderline statistical significance (HR [95% CI] for Q4 versus Q1, 0.84 [0.66–1.07]; P_{Trend}=0.061).[28] This analysis included outcome data through December 31, 2009, while the current analysis includes outcomes occurring over 4 additional years (through December 31, 2013). In another recent analysis from REGARDS, the Mediterranean diet pattern was inversely associated with all-cause mortality[44]; however, this analysis was not restricted to participants with CHD at baseline, as in the current analysis.

There are several mechanisms potentially contributing to an association between greater consumption of the Southern dietary pattern and increased hazard of CHD. Examples include the high sodium content of processed meats that can contribute to hypertension, a risk factor for CHD; nitrite preservatives in processed meats, which have been shown experimentally to promote atherosclerosis and vascular dysfunction[45]; and the high sugar-sweetened beverage consumption characteristic of the Southern pattern, which not only negatively impacts BMI but also increases glycemic load, resulting in insulin resistance, β-cell dysfunction, and inflammation, all setting the stage for atherosclerosis.[46] However, while the Southern pattern was adversely associated with the hazard of incident CHD events in our previous analysis,[18] it was not associated with the hazard of recurrent CHD events in the current analysis. There are several possible explanations for this. First, those participants who experienced a recurrent CHD event may be different in important ways from those who did not, including having different susceptibilities to recognized CHD risk factors, including diet. Second, it is conceivable that risk factors and/or strength of associations vary between incident and recurrent events, such that while an adverse dietary pattern plays an important role in the initiation of CHD, once CHD is established, its importance in the risk factor profile is diminished relative to other concomitant factors. Under either scenario, different covariates would be declared statistically significant between the 2 analyses. Finally, those at greatest risk may have changed their diet to a healthier pattern after their initial CHD event with the intent of reducing the risk of recurrent disease. If this were to occur in a sizable segment of the study population, the risk of recurrent CHD in those adhering to a Southern pattern at baseline might have been underestimated. It is important to note that dietary assessment was conducted only at baseline, so we were unable to assess whether participants changed their dietary patterns between their incident and recurrent CHD events. Changing dietary habits from what are perceived as less healthy to healthier in response to a major life event, such as the diagnosis of an incident CHD event, potentially could have resulted in misclassification of the exposure (dietary intake) in reference to recurrent events, which could have attenuated the results. Unfortunately, we were unable to address this directly in the current study because dietary assessment was not repeated in REGARDS participants.

Possible reasons for disagreement in the results among previous studies noted above include differences in critical study elements such as sample size and population characteristics, dietary assessment instrument and methodology utilized, dietary pattern derivation and selection, and rigorousness of end point determination. The strengths of this study included the large, population-based sample with sociodemographic and regional diversity, including a sizable proportion of black participants, which distinguishes it from most previous studies; a comprehensive assessment of diet with a recognized instrument; derivation of dietary patterns using a rigorous method—factor analysis—based on the actual dietary data collected in the population of interest rather than dietary patterns created a priori based on the authors' opinions on what defines various dietary patterns; and expert adjudication of study end points. Limitations include the recognized potential for measurement error with

any dietary assessment instrument that relies on recall of dietary intake, as is the case with FFQs, which could result in misclassification of dietary intake. However, if random, this would tend to bias results toward the null, potentially reducing the magnitude of the associations between dietary patterns/score and recurrent CHD events and all-cause mortality observed in this analysis. Additionally, those who did not provide a completed FFQ showed no differences in sex or race compared with those who completed the FFQ but were more likely to be black, less educated, and have a lower income. Noncompleters also were slightly more likely to be current smokers and inactive, and had a slightly lower BMI, compared with those who completed the FFQ. In order to reduce potential bias and increase the power of our sample tests, we multiply imputed those who did not complete the FFQ in a sensitivity analysis. Finally, the results observed may not be generalizable to groups other than whites and blacks in the United States.

In summary, the Mediterranean diet score was inversely associated with the hazard of recurrent CHD events and all-cause mortality, while the Southern dietary pattern was associated with the hazard of all-cause mortality but not recurrent CHD events in this diverse sample of white and black adults. Because diet is a modifiable factor, identifying dietary patterns that may contribute to mortality or recurrent CHD events may pave the way for the development of specific nutritional health messages aimed at changing dietary choices made by individuals who have survived CHD.

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Disclosures

None.

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ORIGINAL ARTICLE

Marine n–3 Fatty Acids and Prevention of Cardiovascular Disease and Cancer

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ABSTRACT

BACKGROUND

Higher intake of marine n–3 (also called omega-3) fatty acids has been associated with reduced risks of cardiovascular disease and cancer in several observational studies. Whether supplementation with n–3 fatty acids has such effects in general populations at usual risk for these end points is unclear.

METHODS

We conducted a randomized, placebo-controlled trial, with a two-by-two factorial design, of vitamin D_3 (at a dose of 2000 IU per day) and marine n–3 fatty acids (at a dose of 1 g per day) in the primary prevention of cardiovascular disease and cancer among men 50 years of age or older and women 55 years of age or older in the United States. Primary end points were major cardiovascular events (a composite of myocardial infarction, stroke, or death from cardiovascular causes) and invasive cancer of any type. Secondary end points included individual components of the composite cardiovascular end point, the composite end point plus coronary revascularization (expanded composite of cardiovascular events), site-specific cancers, and death from cancer. Safety was also assessed. This article reports the results of the comparison of n–3 fatty acids with placebo.

RESULTS

A total of 25,871 participants, including 5106 black participants, underwent randomization. During a median follow-up of 5.3 years, a major cardiovascular event occurred in 386 participants in the n–3 group and in 419 in the placebo group (hazard ratio, 0.92; 95% confidence interval [CI], 0.80 to 1.06; P=0.24). Invasive cancer was diagnosed in 820 participants in the n–3 group and in 797 in the placebo group (hazard ratio, 1.03; 95% CI, 0.93 to 1.13; P=0.56). In the analyses of key secondary end points, the hazard ratios were as follows: for the expanded composite end point of cardiovascular events, 0.93 (95% CI, 0.82 to 1.04); for total myocardial infarction, 0.72 (95% CI, 0.59 to 0.90); for total stroke, 1.04 (95% CI, 0.83 to 1.31); for death from cardiovascular causes, 0.96 (95% CI, 0.76 to 1.21); and for death from cancer (341 deaths from cancer), 0.97 (95% CI, 0.79 to 1.20). In the analysis of death from any cause (978 deaths overall), the hazard ratio was 1.02 (95% CI, 0.90 to 1.15). No excess risks of bleeding or other serious adverse events were observed.

CONCLUSIONS

Supplementation with n–3 fatty acids did not result in a lower incidence of major cardiovascular events or cancer than placebo. (Funded by the National Institutes of Health and others; VITAL ClinicalTrials.gov number, NCT01169259.)

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Marine-derived long-chain n–3 (also called omega-3) fatty acids have shown promise for the primary prevention of cardiovascular disease in studies in animals; in small, randomized trials designed with intermediate cardiovascular end points; and in observational epidemiologic investigations.[1] However, midsize-to-large trials testing the effect of n–3 fatty acid supplements on clinical cardiovascular outcomes in the context of secondary prevention or high-risk populations have shown inconsistent results.[1,2] There is a paucity of data from large trials of n–3 supplements for the primary prevention of cardiovascular disease in a general population selected only on the basis of age and not on the basis of cardiovascular risk factors such as diabetes or dyslipidemia. Data from studies of n–3 fatty acids and cancer risk have also been inconsistent.[3] Given the popularity of fish oil as a strategy to reduce the incidence of chronic disease,[4] clarifying the relation between supplemental n–3 fatty acids and risks of cardiovascular disease and cancer and obtaining more-definitive data on the benefit–risk balance of these supplements is a high priority. The Vitamin D and Omega-3 Trial (VITAL) was conducted to address these knowledge gaps in a diverse U.S. cohort.

Methods

Trial Design and Oversight

We conducted this randomized, double-blind, placebo-controlled trial, with a two-by-two factorial design, to test the benefits and risks of supplementation with vitamin D_3 (at a dose of 2000 IU per day) and n–3 fatty acids (1 g per day as a fish-oil capsule containing 840 mg of n–3 fatty acids, including 460 mg of eicosapentaenoic acid [EPA] and 380 mg of docosahexaenoic acid [DHA]) in the primary prevention of cardiovascular disease and cancer among men 50 years of age or older and women 55 years of age or older in the United States. The dose of n–3 fatty acids chosen was the one recommended by the American Heart Association for cardioprotection[5] and shown to be beneficial in a secondary prevention population.[6] The results are presented in two articles, with details of the full trial design provided in the accompanying article containing the vitamin D data,[7] in the Supplementary Appendix (available with the full text of this article at NEJM.org), and in articles that have been published previously.[8,9] The protocol is available at NEJM.org.

Questionnaires were used at baseline to collect data on clinical and lifestyle risk factors and included a dietary questionnaire that ascertained participant-reported intake of fish and other foods. Annual questionnaires assessed adherence to and potential side effects of the randomized trial interventions, the development of major illnesses, and risk-factor updates. Blood samples were obtained at baseline from all willing participants and were assayed for the plasma n–3 index (EPA plus DHA as a percentage of total fatty acids[10]) by Quest Diagnostics with the use of liquid chromatography–tandem mass spectrometry.

The National Institutes of Health, the sponsors of the trial, had a collaborative role in the design and conduct of the trial. Final decisions regarding the data collection, management, and analysis, the review and approval of the manuscript, and the decision to submit the manuscript for publication resided with trial investigators and the trial research group. The trial was approved by the institutional review board of Partners HealthCare–Brigham and Women's Hospital, and the trial agents have received Investigational New Drug approval from the Food and Drug Administration. Pharmavite donated vitamin D and Pronova BioPharma and BASF donated fish oil (Omacor); the companies also donated matching placebos and packaging in the form of calendar packs. Quest Diagnostics measured the plasma n–3 index at no cost to the trial. None of the donating companies had any role in the trial design or conduct, the data collection or analysis, or the manuscript preparation or review. The first three authors and the last author had full access to all the trial data and vouch for the completeness and accuracy of the data, for the accuracy of the data analyses, and for the fidelity of the trial to the protocol. All the participants provided written informed consent before enrollment in the trial.

Trial End Points

The primary end points were major cardiovascular events (composite of myocardial infarction, stroke, and death from cardiovascular causes) and invasive cancer of any type. Secondary cardiovascular end points were major cardiovascular events plus coronary revascularization (percutaneous coronary intervention [PCI] or coronary-artery bypass

grafting [CABG]) and individual components of the primary end point. Secondary cancer end points were colorectal, breast, and prostate cancers during the trial period and death from cancer. Medical records of the participants who had any of the end points were reviewed by an end-points committee of physicians who were unaware of the trial-group assignments. Myocardial infarction and stroke were confirmed with the use of established criteria.[11,12] Cancer was confirmed by histologic or cytologic data.[13] Additional details regarding end-point confirmation are provided in the accompanying article[7] and in the Supplementary Appendix.

Statistical Analysis

Analyses of the effects of the n–3 fatty acid intervention were based on the intention-to-treat principle, as described in the accompanying article on vitamin D supplementation.[7] Primary analyses were based on Cox proportional-hazards models that were controlled for age, sex, and randomization group in the vitamin D portion of the trial (vitamin D group or placebo group).

Possible variations in n–3 treatment effects according to age, sex, baseline cardiovascular risk factors, baseline dietary fish intake and plasma n–3 index, and concurrent randomization to the vitamin D group were specified a priori. Because vitamin D was also studied, the effects in racial or ethnic groups were of interest. Aspirin use and statin use were additional stratification variables. There was no control for multiple hypothesis testing, and no formal adjustment was made to the P values or confidence intervals. Thus, the results regarding exploratory end points and subgroups should be interpreted with caution. Additional details regarding the statistical analyses are provided in the Supplementary Appendix.

Results

Trial Participants

Randomization to receive n–3 fatty acids, vitamin D, both active agents, or both placebos took place from November 2011 through March 2014. The trial intervention ceased as planned on December 31, 2017, which yielded a median follow-up of 5.3 years (range, 3.8 to 6.1). Figure S1 in the Supplementary Appendix shows the enrollment, randomization, and follow-up of the participants.

The characteristics of the trial participants at baseline are shown in Table 1, and in Table S1 in the Supplementary Appendix. Of the 25,871 participants, 51% were women. The mean age of the participants was 67.1 years. The cohort was racially diverse and included 5106 black participants (20.2% of the 25,304 participants with data on race and ethnic group). The characteristics were balanced between the two groups. The rate of response to the questionnaire averaged 93.1%, and rates of adherence to the trial regimen that were reported by the participants (percentage of participants who took at least two thirds of the trial capsules) in the n–3 group averaged 81.6% and in the placebo group averaged 81.5% over 5 years of follow-up (Table S2 in the Supplementary Appendix). The prevalence of outside use of fish-oil supplements was below 3.5% in each group throughout follow-up.

Blood samples were obtained at baseline from 16,956 of 25,871 participants (65.5%). Among the 15,535 participants (60.0%) who had blood samples at baseline that could be analyzed, the mean (±SD) plasma n–3 index was 2.7±0.9% in each group. Among the 1583 participants who also provided a blood sample at 1 year that could be analyzed, the mean n–3 index rose to 4.1% (increase of 54.7%) in the n–3 group and changed by less than 2% in the placebo group. There was no interaction between the two active treatments in the two-by-two factorial trial design. The outcomes regarding vitamin D supplementation are presented in the accompanying article.[7]

Cardiovascular Disease

During follow-up, there were 805 major cardiovascular events, with events in 386 participants in the n–3 group and in 419 in the placebo group (hazard ratio, 0.92; 95% confidence interval [CI], 0.80 to 1.06; P=0.24) (Table 2). In the analyses of prespecified secondary cardiovascular end points, the hazard ratios were as follows: for total myocardial infarction, 0.72 (95% CI, 0.59 to 0.90); for death from cardiovascular causes, 0.96 (95% CI, 0.76 to 1.21); for total stroke, 1.04 (95% CI, 0.83 to 1.31); and for the expanded composite end point of cardiovascular events, 0.93 (95% CI, 0.82 to 1.04). Additional cardiovascular end points included PCI (hazard ratio, 0.78; 95% CI, 0.63 to 0.95), CABG (hazard ratio, 0.99; 95% CI, 0.73 to 1.33), fatal myocardial infarction (hazard ratio, 0.50; 95% CI, 0.26 to 0.97), and total coronary heart disease (hazard ratio, 0.83; 95% CI, 0.71 to 0.97) (Ta-

Table 1. Characteristics of the Participants at Baseline, According to Randomized Assignment to Marine n–3 Fatty Acids or Placebo.*

Characteristic	Total (N=25,871)	n–3 Group (N=12,933)	Placebo Group (N=12,938)
Age — yr	67.1±7.1	67.2±7.1	67.1±7.1
Female sex — no. (%)	13,085 (50.6)	6547 (50.6)	6538 (50.5)
Race or ethnic group — no./total no. (%)†			
Non-Hispanic white	18,046/25,304 (71.3)	9044/12,653 (71.5)	9002/12,651 (71.2)
Black	5106/25,304 (20.2)	2549/12,653 (20.1)	2557/12,651 (20.2)
Nonblack Hispanic	1013/25,304 (4.0)	491/12,653 (3.9)	522/12,651 (4.1)
Asian or Pacific Islander	388/25,304 (1.5)	200/12,653 (1.6)	188/12,651 (1.5)
Native American	228/25,304 (0.9)	120/12,653 (0.9)	108/12,651 (0.9)
Other or unknown	523/25,304 (2.1)	249/12,653 (2.0)	274/12,651 (2.2)
Body-mass index‡	28.1±5.7	28.1±5.7	28.1±5.8
Current smoking — no./total no. (%)	1836/25,485 (7.2)	920/12,739 (7.2)	916/12,746 (7.2)
Hypertension treated with medication — no./total no. (%)	12,791/25,698 (49.8)	6338/12,853 (49.3)	6453/12,845 (50.2)
Current use of cholesterol-lowering medication — no./total no. (%)	9524/25,428 (37.5)	4788/12,707 (37.7)	4736/12,721 (37.2)
Diabetes — no./total no. (%)	3549/25,828 (13.7)	1799/12,912 (13.9)	1750/12,916 (13.5)

* Plus–minus values are means ±SD. There were no significant differences between the two groups with regard to the baseline characteristics. Percentages may not total 100 because of rounding.
† Race and ethnic group were reported by the participant.
‡ The body-mass index is the weight in kilograms divided by the square of the height in meters. Data were available for 12,615 participants in the n–3 group and for 12,639 in the placebo group.

ble 2). The results regarding stroke subtypes and death from stroke are shown in Table 2.

Cumulative incidence rates of major cardiovascular events are shown in Figure 1A. For major cardiovascular events, the curves did not differ significantly between the two groups. In an analysis that excluded the first 2 years of follow-up, the hazard ratio for major cardiovascular events in the n–3 group, as compared with the placebo group, was 0.89 (95% CI, 0.76 to 1.05), and the lower incidence of myocardial infarction in the n–3 group persisted (Table 2). The cumulative incidence rates of the prespecified secondary end points are shown in Figure S2 in the Supplementary Appendix.

Subgroup analyses showed a possible lower incidence of the primary cardiovascular end point with n–3 supplementation than with placebo among participants with low fish consumption (Fig. 2). Additional subgroup analyses are presented in Tables S3 and S4 and Figure S3 in the Supplementary Appendix, with a focus on exploring differences according to racial or ethnic group, diabetes status, number of traditional cardiovascular risk factors, dietary fish intake, and other variables for the primary end point of major cardiovascular events and the secondary end point of total myocardial infarction. For myocardial infarction, these analyses are presented as explanatory analyses to assess whether the effect of the intervention was similar across subgroups. The suggestion of greater differences in the risk of myocardial infarction among blacks and among those with low fish intake, comparing the n–3 group with the placebo group, is discussed in the Supplementary Appendix. For the other secondary cardiovascular end points of stroke, death from cardiovascular causes, and the expanded composite of major cardiovascular events plus coronary revascularization, no appreciable effect modification was found (data not shown).

CANCER AND ALL-CAUSE MORTALITY

During follow-up, invasive cancer developed in 1617 participants (820 in the n–3 group vs. 797 in the placebo group), with similar risks in the two groups (hazard ratio, 1.03; 95% CI, 0.93 to 1.13; P=0.56) (Table 2). No significant differences between the randomized groups were observed with regard to the incidence of breast, prostate, or

Table 2. Hazard Ratios and 95% Confidence Intervals for the Primary, Secondary, and Other End Points, According to Randomized Assignment to n–3 Fatty Acids or Placebo, in Intention-to-Treat Analyses.*

End Point	n–3 Group (N = 12,933)	Placebo Group (N = 12,938)	Hazard Ratio (95% CI)
	no. of participants with event		
Cardiovascular disease			
Primary end point: major cardiovascular event†	386	419	0.92 (0.80–1.06)
Cardiovascular event in expanded composite end point‡	527	567	0.93 (0.82–1.04)
Total myocardial infarction	145	200	0.72 (0.59–0.90)
Total stroke	148	142	1.04 (0.83–1.31)
Death from cardiovascular causes	142	148	0.96 (0.76–1.21)
Other cardiovascular end point§			
PCI	162	208	0.78 (0.63–0.95)
CABG	85	86	0.99 (0.73–1.33)
Total coronary heart disease¶	308	370	0.83 (0.71–0.97)
Ischemic stroke	111	116	0.96 (0.74–1.24)
Hemorrhagic stroke	25	19	1.32 (0.72–2.39)
Death from coronary heart disease	37	49	0.76 (0.49–1.16)
Death from myocardial infarction	13	26	0.50 (0.26–0.97)
Death from stroke	22	20	1.10 (0.60–2.01)
Cancer			
Primary end point: invasive cancer of any type	820	797	1.03 (0.93–1.13)
Breast cancer	117	129	0.90 (0.70–1.16)
Prostate cancer	219	192	1.15 (0.94–1.39)
Colorectal cancer	54	44	1.23 (0.83–1.83)
Death from cancer	168	173	0.97 (0.79–1.20)
Death from any cause	493	485	1.02 (0.90–1.15)
Analyses excluding the first 2 yr of follow-up			
Major cardiovascular event	269	301	0.89 (0.76–1.05)
Total myocardial infarction	94	131	0.72 (0.55–0.93)
Invasive cancer of any type	536	476	1.13 (1.00–1.28)
Death from cancer	126	135	0.93 (0.73–1.19)
Death from any cause	371	381	0.97 (0.84–1.12)

* Analyses were from Cox regression models that were controlled for age, sex, and randomization group in the vitamin D portion of the trial. The 95% confidence intervals were not adjusted for multiple comparisons.
† This end point was a composite of myocardial infarction, stroke, or death from cardiovascular causes.
‡ This end point was a composite of myocardial infarction, stroke, death from cardiovascular causes, or coronary revascularization (percutaneous coronary intervention [PCI] or coronary-artery bypass grafting [CABG]).
§ These events were not prespecified as primary or secondary end points.
¶ This end point was a composite of myocardial infarction, coronary revascularization (PCI or CABG), and death from coronary heart disease.

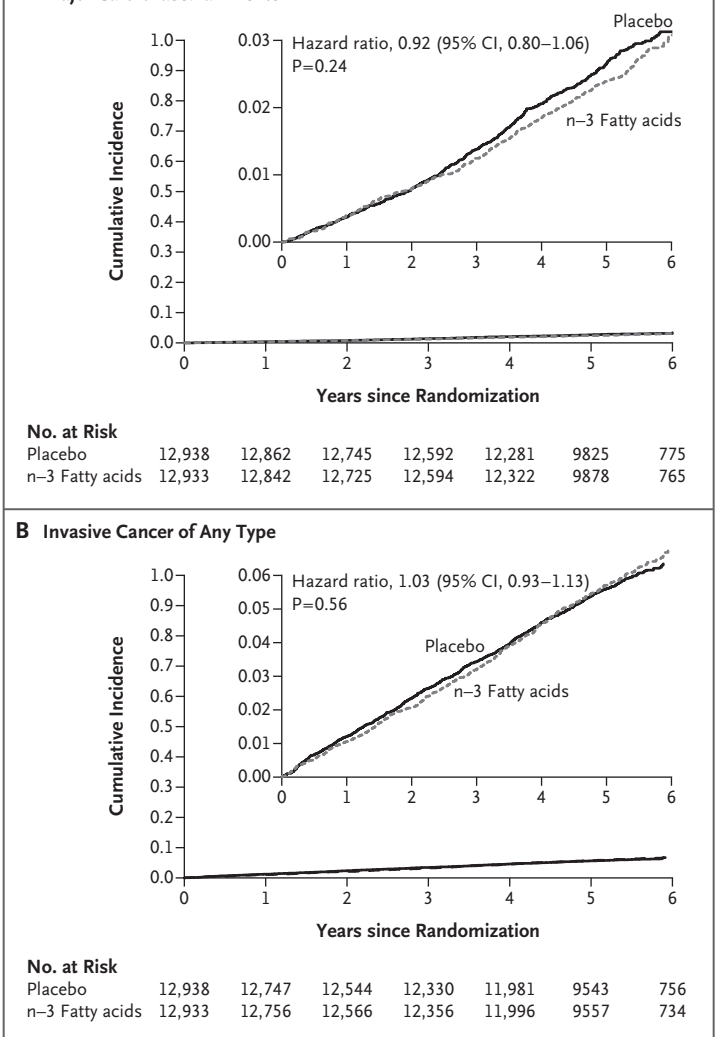

Figure 1. Cumulative Incidence Rates of Major Cardiovascular Events and Invasive Cancer of Any Type, According to Year of Follow-up, in the n–3 Group and the Placebo Group.

Analyses were from Cox regression models that were controlled for age, sex, and randomization group in the vitamin D portion of the trial (intention-to-treat analyses). The insets show the same data on an enlarged y axis.

colorectal cancer; death from cancer (341 deaths from cancer; hazard ratio, 0.97; 95% CI, 0.79 to 1.20); or death from any cause (978 deaths overall; hazard ratio, 1.02; 95% CI, 0.90 to 1.15).

The cumulative-incidence curves for cancer did not differ significantly between the trial groups at any year of follow-up (Fig. 1B). Tests for proportionality over time in the proportional-hazards analysis suggested violation for cancer (P=0.08). In analyses that excluded the first 2 years of follow-up, the hazard ratio for cancer in the n–3 group, as compared with the placebo group, was 1.13 (95% CI, 1.00 to 1.28), and the hazard ratio for death from cancer was 0.93 (0.73 to 1.19) (Table 2).

In the subgroup analyses, the variable of sex may have modified the results regarding cancer incidence (P=0.02 for interaction) (Table S5 in the Supplementary Appendix). Fish intake at baseline may have modified the effects of the intervention on the incidence of death from any cause (P=0.02 for interaction) (Table S6 in the Supplementary Appendix). There were no other significant interactions regarding cancer end points or death from any cause.

ADVERSE EVENTS

The incidence of gastrointestinal symptoms, major bleeding episodes, or other serious adverse events did not differ significantly between the n–3 group and the placebo group. Details are provided in Table S7 in the Supplementary Appendix.

DISCUSSION

In this primary prevention trial with a median follow-up of 5.3 years, supplementation with n–3 fatty acids at a dose of 1 g per day did not lead to a significantly lower incidence of the primary end points of major cardiovascular events (a composite of myocardial infarction, stroke, and death from cardiovascular causes) or invasive cancer than placebo. Analyses of the components of the primary composite cardiovascular end point suggested that the risk of myocardial infarction was lower in the n–3 group than in the placebo group and that there was no significant difference in the incidence of death from cardiovascular causes or stroke. Exploratory analyses that excluded the first 2 years of follow-up suggested a nonsignificantly higher incidence of cancer in the n–3 group than in the placebo group but not a higher incidence of death from cancer.

Meta-analyses of n–3 supplementation trials involving adults who had cardiovascular disease or who were at high risk for cardiovascular disease have shown that supplementation has no, or at most a weak, preventive effect on cardiovascu-

Subgroup	No. of Participants	n–3 Fatty Acids	Placebo	Hazard Ratio (95% CI)	P Value for Interaction
		no. of participants with event			
Age	25,871				0.84
<Median of 66.7 yr	12,859	129	142	0.91 (0.71–1.15)	
≥Median of 66.7 yr	13,012	257	277	0.93 (0.78–1.10)	
Sex	25,871				0.88
Male	12,786	213	233	0.91 (0.76–1.10)	
Female	13,085	173	186	0.93 (0.76–1.15)	
Race	25,304				0.26
Non-Hispanic white	18,046	292	289	1.00 (0.85–1.18)	
Black	5,106	62	83	0.74 (0.53–1.03)	
Other	2,152	26	30	0.94 (0.55–1.59)	
Current smoker	25,485				0.77
No	23,649	340	365	0.93 (0.80–1.07)	
Yes	1,836	41	44	0.94 (0.62–1.44)	
Diabetes	25,860				0.19
No	23,132	334	349	0.96 (0.82–1.11)	
Yes	2,728	52	70	0.74 (0.51–1.05)	
Hypertension	25,698				0.32
No	12,907	151	147	1.01 (0.80–1.26)	
Yes	12,791	231	270	0.87 (0.73–1.03)	
Current cholesterol medication	25,428				0.77
No	15,904	236	252	0.94 (0.79–1.13)	
Yes	9,524	140	154	0.90 (0.72–1.13)	
Parental history of myocardial infarction	22,915				0.56
No	19,262	268	288	0.93 (0.79–1.10)	
Yes	3,653	61	71	0.83 (0.59–1.17)	
Fish consumption	25,435				0.045
<Median of 1.5 servings/wk	13,514	189	232	0.81 (0.67–0.98)	
≥Median of 1.5 servings/wk	11,921	189	176	1.08 (0.88–1.32)	
Randomization in vitamin D portion of trial	25,871				0.56
Placebo group	12,944	200	209	0.96 (0.79–1.16)	
Vitamin D group	12,927	186	210	0.88 (0.72–1.08)	
No. of cardiovascular risk factors	25,871				0.19
0	7,802	92	85	1.06 (0.79–1.42)	
1	8,948	133	141	0.95 (0.75–1.20)	
≥2	9,121	161	193	0.84 (0.68–1.04)	
Baseline aspirin use	25,497				0.68
No	13,927	192	199	0.96 (0.78–1.17)	
Yes	11,570	187	209	0.90 (0.74–1.10)	
Baseline statin use	25,447				0.57
No	16,557	247	260	0.95 (0.80–1.14)	
Yes	8,890	130	147	0.88 (0.69–1.11)	

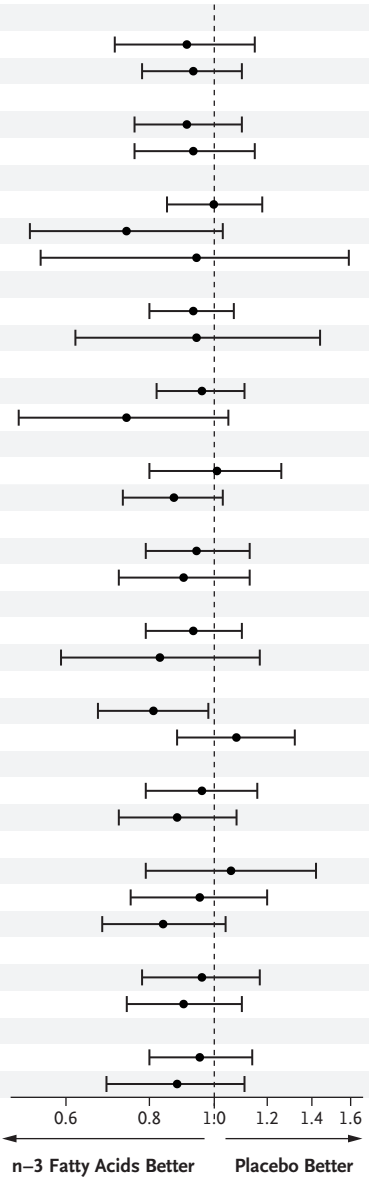

Figure 2. Hazard Ratios and 95% Confidence Intervals of Major Cardiovascular Events According to Subgroup, Comparing the n–3 Group with the Placebo Group.

Analyses were from Cox regression models that were controlled for age, sex, and randomization group in the vitamin D portion of the trial (intention-to-treat analyses). Analyses were not adjusted for multiple comparisons. Race and ethnic group were reported by the participant. Participants with diabetes and hypertension were defined as those receiving treatment for each condition. Parental history of myocardial infarction was defined as early myocardial infarction in a parent (at <60 years of age in father or <65 years of age in mother). Cardiovascular risk factors were smoking, diabetes, hypertension, a high cholesterol level, and parental history of early myocardial infarction.

lar outcomes, including major cardiovascular events, major coronary events, myocardial infarction, stroke, and revascularization.[14-16] The recent ASCEND (A Study of Cardiovascular Events in Diabetes) trial,[17] which tested n–3 supplementation (at a dose of 1 g per day) in adults with diabetes in the United Kingdom, also showed generally null results. Thus, the possible benefit of the intervention with respect to the secondary end points of myocardial infarction and PCI in our trial, which tested n–3 fatty acids for primary prevention in a usual-risk population, raises the question of potential differences between results from primary and secondary prevention trials. Neither our trial nor the secondary prevention trials indicate a benefit of n–3 supplementation with respect to stroke or composite cardiovascular end points. Our finding of a possible lower incidence of the primary cardiovascular end point with n–3 supplementation than with placebo among participants with low fish consumption — a characteristic that has rarely been examined as an effect modifier in previous trials — is hypothesis-generating.

Two early, large, open-label trials that involved more than 10,000 participants[6,18] and tested doses of 1 g or more of n–3 fatty acids per day showed significant protection against coronary events. However, all but one[19] of the subsequent placebo-controlled trials[17,19-24] (some with smaller sample sizes[19-22] and lower doses[20,22]) did not. The finding of a lower risk of coronary events with n–3 fatty acids than with placebo in our trial may be attributable in part to these design differences. Also, the prevalence of the use of medications for cardiovascular disease, including statins, beta-blockers, and anticoagulants, was higher in recent trials than in our trial, perhaps reducing the opportunity to detect incremental benefit. Although a recent meta-analysis[15] of n–3 trials showed no variation in results according to statin use, the dilution of a potential effect of n–3 supplementation by other medications cannot be ruled out. Such a dilution would probably be greater in the context of secondary prevention, in which medication use is more prevalent than in the context of primary prevention. In addition, participants in secondary prevention trials generally have more advanced atherosclerosis than those in primary prevention trials, which may necessitate the use of more powerful interventions than n–3 fatty acids (or higher doses of n–3 fatty acids) to avert clinical events. Indeed, a greater benefit of n–3 supplementation on major cardiovascular events was observed among participants without a history of stroke in a recent meta-analysis[15] and among those without a history of cardiovascular disease in a trial involving patients with macular degeneration[25] than among those with such histories. Differences in fish consumption across study populations may have also influenced findings. Finally, there were few black participants in the secondary prevention trials, and our trial suggests that there is a greater coronary benefit of supplemental n–3 fatty acids in this racial group than in others.

The finding in subgroup analyses of the secondary end point of myocardial infarction that suggested possible greater cardiovascular benefits of n–3 supplementation in blacks than in non-Hispanic whites was unexpected, especially given that both these racial and ethnic groups had similar blood levels of EPA and DHA at baseline and similar fish intake. It may be a chance finding that would require corroboration in future trials. Recent observational studies have shown racial variation in associations of both marine and plant-derived n–3 biomarkers with the incidence of coronary disease.[26] Gene variants influence metabolism and the bioavailability of n–3 fatty acids, as has been observed in Greenland Inuits,[27] and may influence coronary risk.[28] Other racial and ethnic differences in clinical, dietary, or environmental factors may also account for this finding.[29] Finally, blacks have a higher prevalence of coexisting conditions such as diabetes and hypertension than do non-Hispanic whites. However, treatment-associated hazard ratios for myocardial infarction were lower across cardiovascular-risk strata among blacks, with lower hazard ratios than among non-Hispanic whites (Table S3 in the Supplementary Appendix).

The hypothesis that supplemental n–3 fatty acids confer coronary protection is biologically plausible. Data from laboratory studies and from studies in animals, as well as from small trials of intermediate cardiovascular end points in humans, support mechanisms including antithrombotic, hypotriglyceridemic, blood-pressure–lowering, and antiinflammatory effects; impeded growth of atherosclerotic plaques; slowing of heart rate; reduced susceptibility to cardiac arrhythmias; and the promotion of nitric oxide–induced endo-

thelial relaxation whereby n–3 fatty acids may reduce risk.[1,8] Data from experimental studies provide support for relevant molecular and gene-regulatory effects.[1] The dose–response curve for most effects plateaus at 1 g or less of n–3 fatty acids per day.[30] Observational studies suggest significant inverse associations between fish intake or n–3 fatty acid biomarkers and coronary outcomes — findings that are compatible with these mechanisms.[26,31-33]

With regard to cancer, our findings are consistent with the results of secondary prevention trials of n–3 fatty acids for cardiovascular disease, which have mostly shown neutral effects or slight (but nonsignificant) elevations in cancer incidence with n–3 fatty acids.[6,17,18,23,24,34,35] A 2014 meta-analysis of 10 trials of n–3 fatty acids showed a risk of cancer that was nonsignificantly higher, by 10%, with the n–3 fatty acids than with placebo (P=0.12).[36] A 2018 meta-analysis of n–3 trials of cardiovascular disease[15] also showed no significant association between supplementation and incidence of cancer but did not provide an effect estimate. Our finding of a more favorable effect regarding the incidence of cancer among women contrasts with the results of a European trial of n–3 fatty acids,[34] which showed a higher risk of cancer with n–3 fatty acids than with placebo among women but not among men. Among three trials investigating cancer mortality, two have shown a neutral treatment effect on the rate of death from cancer[17,19] and one has shown a possible benefit.[35] The lack of a significant treatment effect of n–3 supplementation on all-cause mortality in the present trial is consistent with the results of meta-analyses of earlier trials[14,16] and with the results of ASCEND.[17]

The strengths of our trial include a large general population sample with racial, ethnic group, and geographic diversity; high rates of follow-up and adherence to the pill regimen; high rates of obtaining blood samples; validated biomarkers of adherence to the regimen; dietary assessments; and rigorously adjudicated end points. Ancillary studies examining diabetes, atrial fibrillation, cognition, autoimmune disorders, and other outcomes in our trial are in progress and may inform the overall benefit–risk balance of n–3 supplementation.

Our trial also has certain limitations. The median duration of the trial intervention was 5.3 years. The single dose level of n–3 fatty acids that was used in this trial did not permit exploration of dose–response relationships. However, the dose that we used has been recommended by the American Heart Association for cardioprotection in persons with a history of coronary disease[5,37] and is at least twice the dose that has been recommended for cardiovascular protection in healthy populations (equivalent to 1 to 2 servings of fish per week).[31,37] The results of ongoing trials[38,39] that are testing higher doses in high-risk populations will be informative but may not apply to primary prevention. Some of our subgroup analyses are based on small numbers of events.

In conclusion, supplementation with n–3 fatty acids did not result in a lower incidence than placebo of the primary end points of major cardiovascular events (a composite of myocardial infarction, stroke, or death from cardiovascular causes) and invasive cancer of any type.

The views expressed in this article are those of the authors and do not necessarily represent the views of the Department of Health and Human Services or the National Institutes of Health.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

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JAMA Cardiology | Original Investigation

Associations of Omega-3 Fatty Acid Supplement Use With Cardiovascular Disease Risks

Meta-analysis of 10 Trials Involving 77 917 Individuals

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IMPORTANCE Current guidelines advocate the use of marine-derived omega-3 fatty acids supplements for the prevention of coronary heart disease and major vascular events in people with prior coronary heart disease, but large trials of omega-3 fatty acids have produced conflicting results.

OBJECTIVE To conduct a meta-analysis of all large trials assessing the associations of omega-3 fatty acid supplements with the risk of fatal and nonfatal coronary heart disease and major vascular events in the full study population and prespecified subgroups.

DATA SOURCES AND STUDY SELECTION This meta-analysis included randomized trials that involved at least 500 participants and a treatment duration of at least 1 year and that assessed associations of omega-3 fatty acids with the risk of vascular events.

DATA EXTRACTION AND SYNTHESIS Aggregated study-level data were obtained from 10 large randomized clinical trials. Rate ratios for each trial were synthesized using observed minus expected statistics and variances. Summary rate ratios were estimated by a fixed-effects meta-analysis using 95% confidence intervals for major diseases and 99% confidence intervals for all subgroups.

MAIN OUTCOMES AND MEASURES The main outcomes included fatal coronary heart disease, nonfatal myocardial infarction, stroke, major vascular events, and all-cause mortality, as well as major vascular events in study population subgroups.

RESULTS Of the 77 917 high-risk individuals participating in the 10 trials, 47 803 (61.4%) were men, and the mean age at entry was 64.0 years; the trials lasted a mean of 4.4 years. The associations of treatment with outcomes were assessed on 6273 coronary heart disease events (2695 coronary heart disease deaths and 2276 nonfatal myocardial infarctions) and 12 001 major vascular events. Randomization to omega-3 fatty acid supplementation (eicosapentaenoic acid dose range, 226-1800 mg/d) had no significant associations with coronary heart disease death (rate ratio [RR], 0.93; 99% CI, 0.83-1.03; $P = .05$), nonfatal myocardial infarction (RR, 0.97; 99% CI, 0.87-1.08; $P = .43$) or any coronary heart disease events (RR, 0.96; 95% CI, 0.90-1.01; $P = .12$). Neither did randomization to omega-3 fatty acid supplementation have any significant associations with major vascular events (RR, 0.97; 95% CI, 0.93-1.01; $P = .10$), overall or in any subgroups, including subgroups composed of persons with prior coronary heart disease, diabetes, lipid levels greater than a given cutoff level, or statin use.

CONCLUSIONS AND RELEVANCE This meta-analysis demonstrated that omega-3 fatty acids had no significant association with fatal or nonfatal coronary heart disease or any major vascular events. It provides no support for current recommendations for the use of such supplements in people with a history of coronary heart disease.

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Supplemental content

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Observational studies in Western and Asian populations have reported that regular consumption of fish once or twice a week is associated with lower risks of death from coronary heart disease (CHD).[1,2] These observations, together with the lower rates of CHD in populations that consumed large amount of foods rich in very-long-chain polyunsaturated fatty acids containing omega-3 fatty acids have prompted interest in assessing whether consumption of marine-derived very-long-chain omega-3 fatty acids (abbreviated "omega-3 FA" in this article) may be protective for CHD.[3] These marine-derived omega-3 FAs include eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) found in fish and other seafood, but not alpha-linolenic acid, which is plant-derived.

The initial Diet and Reinfarction Trial-1 study[4] examined the associations of consumption of oily fish twice or more per week with CHD risk in men who had had a myocardial infarction and reported that fish consumption was associated with a significant reduction in fatal CHD and all-cause mortality but had no association with nonfatal myocardial infarction (MI) recurrence.[4] However, the subsequent Diet and Reinfarction Trial-2 study in men with angina reported that consumption of fish or fish oil supplements increased the risk of CHD death.[5] Subsequently, several large trials have reported conflicting results of the associations of supplementation with omega-3 FA supplements vs placebo or untreated controls on fatal and nonfatal vascular events.[6-16]

Ten large randomized trials[6-15] have been conducted comparing the associations of treatment with omega-3 FA supplementation vs placebo or no treatment for at least 12 months in populations with prior CHD, stroke, or high risk of cardiovascular disease (CVD). These trials have reported conflicting results for the associations of treatment with fatal CHD, nonfatal CHD, or other subtypes of CVD. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial,[6] an open-label trial involving 11 323 recent survivors of MI, reported that patients who received supplementation with omega-3 FAs experienced a 10% reduced risk of major cardiovascular events compared with untreated controls. The Japan EPA Lipid Intervention Study (JELIS) trial, an open-label trial involving 18 645 participants with total cholesterol of 243.24 mg/dL (to convert to mmol/L, multiply by 0.0259) or greater, of whom only 20% with prior CHD, also reported[14] that supplementation with fish oil was associated with a 19% reduction in major CHD events (95% CI, 5%-31%). None of the other large placebo-controlled trials reported any significant association with CHD or mortality. Hence, it is unclear whether the discrepant results reflect different associations of omega-3 FAs with CHD subtypes, different outcomes in primary vs secondary prevention of CHD, increasing use of statins with better control of lipid levels, or an artifact of chance or bias in open-label trials. Previous meta-analyses of these trials of omega-3 FA supplements[16-18] appeared to suggest a significant beneficial association of omega-3 FAs with fatal CHD but not nonfatal CHD. However, these meta-analyses were constrained because they included trials of dietary advice to eat fish[17] or excluded trials that did not include a placebo control.[18]

The Omega-3 Treatment Trialists' Collaboration was established to conduct a collaborative meta-analysis based on aggregated study-level data obtained from the principal investigators of all large randomized clinical trials of omega-3 FA supplements for the prevention of cardiovascular disease, using a prespecified protocol and analysis plan. The aims of this meta-analysis were to assess the associations of supplementation with omega-3 FAs on (1) fatal CHD, nonfatal MI, stroke, major vascular events, and all-cause mortality and (2) major vascular events in prespecified subgroups.

Key Points

Question Does supplementation with marine-derived omega-3 fatty acids have any associations with reductions in fatal or nonfatal coronary heart disease in people at high risk of cardiovascular disease?

Findings This meta-analysis of 10 trials involving 77 917 participants demonstrated that supplementation with marine-derived omega-3 fatty acids for a mean of 4.4 years had no significant association with reductions in fatal or nonfatal coronary heart disease or any major vascular events.

Meaning The results provide no support for current recommendations to use omega-3 fatty acid supplements for the prevention of fatal coronary heart disease or any cardiovascular disease in people who have or at high risk of developing cardiovascular disease.

Methods

We performed a systematic search of randomized clinical trials in PubMed and Medline data sets, supplemented by manual hand-searching of reference lists from individual trials, review articles, or previous meta-analyses of omega-3 FAs and CVD (eFigure 1 in the Supplement). Search terms included "omega-3 FA," "omega-3 polyunsaturated fat," "fish oils," and "ω-3 FA" and "cardiovascular disease" or "coronary heart disease" or "stroke" (eFigure 1 in the Supplement). The prespecified eligibility criteria were randomized clinical trials of marine-derived very-long-chain omega-3 FA supplements vs placebo or open-label control, with a sample size of at least 500 participants and a scheduled duration of treatment of at least 1 year. All eligible trials required use of supplements, but no minimum daily dose of EPA or DHA was specified. The prespecified end points included nonfatal MI; death caused by CHD; ischemic, hemorrhagic, and unclassified stroke; coronary or noncoronary arterial revascularization events; major vascular events (a composite of first occurrence of nonfatal MI or death caused by CHD; nonfatal or fatal stroke; or any revascularization procedure); and all-cause mortality. Deaths caused by CHD included sudden cardiac deaths, deaths due to ventricular arrhythmias, and heart failure in patients with CHD, MI, or deaths occurring after coronary revascularization or heart transplant.

All included trials were also assessed for risk of bias. Individual trials had approval from their respective institutional review boards, and all participants provided written informed consent. No additional ethical approval was required for this meta-analysis.

Table. Characteristics of Included Trials

Study (Year)	Patients, No.	Dose of EPA/ DHA (mg/d)	Male, No. (%)	Mean Trial Duration, y	Mean (SD) Age, y	No. (%)			
						Prior CHD	Prior Stroke	Prior Diabetes	Statin Use
DOIT (2010)	563	1150/800	563 (100)	3	70 (3)	133 (23.6)	37 (6.6)	46 (8.2)	NA
AREDS-2 (2014)	4203	650/350	1816 (43.2)	4.5	74 (NA)	405 (9.7)	211 (5.0)	546 (13.0)	1866 (44.4)
SU.FOL.OM3 (2010)	2501	400/200	1987 (79.4)	4.7	61 (NA)	1863 (74.5)	638 (25.5)	440 (17.9)	2079 (83.1)
JELIS (2007)[a,b]	18 645	1800/NA	5859 (31.4)	4.6	61 (8)	NA	NA	3040 (16.3)	18 645 (100.0)
Alpha Omega (2010)	4837	226/150	3783 (78.2)	3.3	69 (6)	4837 (100.0)	345 (7.2)	1014 (21.0)	4122 (85.2)
OMEGA (2010)	3818	460/380	2841 (74.4)	1	64 (NA)	796 (22.5)	192 (5.5)	948 (27.0)	3566 (94.2)
R&P (2013)	12 505	500/500	7687 (61.5)	5	64 (NA)	Not stated (30)	594 (4.8)	7494 (59.9)	12 505 (100.0)
GISSI-HF (2008)	6975	850/950	5459 (78.3)	3.9	67 (11)	3614 (51.8)	346 (5.0)	1974 (28.3)	NA
ORIGIN (2012)	12 536	465/375	8150 (65.0)	6.2	64 (8)	8094 (64.6)	10 877 (86.8)	11 081 (88.4)	6739 (53.8)
GISSI-P[b] (1999)	11 334	850/1700	9658 (85.2)	3.5	59 (11)	11 334 (100.0)	NA	2139 (18.9)	NA
Total	77 917	NA	47 803 (61.4)	4.4	64	31 076/46 767 (66.4)	13 240/47 938 (27.6)	28 722 (36.9)	49 522 (83.4)

Abbreviations: AREDS-2, Age-Related Eye Disease Study 2; DOIT, Diet and Omega-3 Intervention Trial; GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Heart Failure; GISSI-P, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione; JELIS, Japan Eicosapentaenoic Acid (EPA) Lipid Intervention Study; NA, not available; OMEGA, Effect of Omega 3-Fatty Acids on the Reduction of Sudden Cardiac Death After Myocardial Infarction; ORIGIN, Outcome Reduction With Initial Glargine Intervention; SU.FOL.OM3, Supplémentation en Folates et Omega-3; R&P, Risk and Prevention Study.

[a] All trials used eicosapentaenoic acid and docosahexanoic acid supplements, with the exception of the JELIS trial (eicosapentaenoic acid only).

[b] All trials were blind, placebo-controlled randomized clinical trials with the exception of JELIS and GISSI-P, which were open-label without placebo.

A protocol outlining the eligibility criteria, prespecified analyses, and plans for publication together with standardized data request forms were sent to the principal investigators of all eligible trials. The study used the PRISMA guidelines for the conduct of meta-analysis of randomized trials.[19] Aggregated study-level (tabular) data were successfully obtained from 9 of the 10 trials (**Table**; eTable in the Supplement).[6-13,15] The JELIS trial[14] declined to participate in this collaboration, but the published results of the trial were sufficiently detailed to allow its inclusion in this study. Any discrepancies between data supplied and the published reports were clarified by contacting trial investigators.

Statistical Analysis

The association of treatment with outcomes in each trial was analyzed separately, and summary statistics were calculated for each trial. For each trial, we calculated the observed minus expected statistic (O−E) and its variance (V) from the number of patients who developed the relevant end point and the total number of patients in each treatment group, using standard formulas for 2 × 2 contingency tables. One O−E value from each trial was summed to produce a grand total (G), with variance (V) equal to the sum of their separate variances. The value exp(G/V) is Peto 1-step estimate of the rate ratio (RR), and its continuity-corrected 95% confidence interval is given by $\exp(G/V \pm [0.5/V + (1.96/\sqrt{V})])$.[20] Rate ratios are given with 95% CI for the overall results for major diseases and with 99% CI (which is calculated by replacing 1.96 in the formula above by 2.58) for the results of individual trials or subgroups of trials or subgroups of such major diseases. Heterogeneity between the different subgroups is assessed by first calculating $S-(G^2/V)$, where S is the sum of $(O-E)^2/V$ for each trial (or subgrouping), and then testing this statistic against a χ^2 distribution with the degrees of freedom equal to 1 fewer than the number of subgroups. The meta-analysis was repeated after excluding the JELIS trial,[14] since it tested EPA alone rather than the combination of EPA and DHA used in all other trials.[6-13,15]

Additional analyses of the primary outcomes assessed the associations of treatment with major vascular events in predefined subgroups, including age, sex, prior CHD, prior stroke, prior diabetes, blood lipids (total cholesterol, triglyceride, high-density lipoprotein, and calculated or measured low-density lipoprotein), prior use of statins, and trial design (open-label or blinded). In interpreting subgroup results, the chief emphasis was placed on the overall results, unless there was strong evidence of heterogeneity ($P < .001$). Sensitivity analyses compared the results of the Peto method with log-rank method in the 1 trial that had also provided individual participant data on all events.

Results

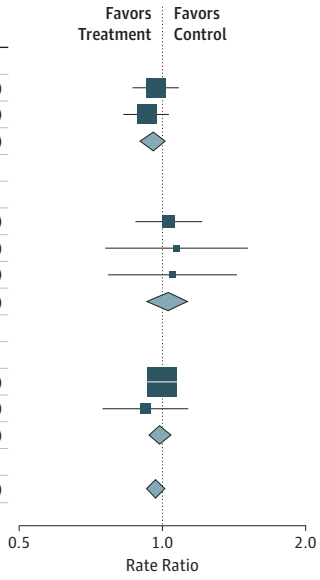
Characteristics of Individual Trials

Study level data were obtained on a total of the 10 trials[6-15] that met the inclusion criteria. A total of 77 917 participants were involved, and trials ranged in size from 563 to 18 645 participants (Table; eTable in the Supplement). Of the 10 trials, 8 had a double-blind design and used a placebo control, and 2 trials had an open-label design.[6,14] The risk of bias of the included trials was low, with exception of the 2 trials that did not use a placebo-treated control group[6,14] (eFigure 2 in the Supplement).

Combinations of polyunsaturated fatty acid ethyl esters of EPA and DHA were used in all but 1 trial,[14] which tested daily

Figure 1. Associations of Omega-3 Fatty Acids With Major Vascular Events

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Coronary heart disease			
Nonfatal myocardial infarction	1121 (2.9)	1155 (3.0)	0.97 (0.87-1.08)
Coronary heart disease death	1301 (3.3)	1394 (3.6)	0.93 (0.83-1.03)
Any	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12
Stroke			
Ischemic	574 (1.9)	554 (1.8)	1.03 (0.88-1.21)
Hemorrhagic	117 (0.4)	109 (0.4)	1.07 (0.76-1.51)
Unclassified/other	142 (0.4)	135 (0.3)	1.05 (0.77-1.43)
Any	870 (2.2)	843 (2.2)	1.03 (0.93-1.13)
			P = .60
Revascularization			
Coronary	3044 (9.3)	3040 (9.3)	1.00 (0.93-1.07)
Noncoronary	305 (2.7)	330 (2.9)	0.92 (0.75-1.13)
Any	3290 (10.0)	3313 (10.2)	0.99 (0.94-1.04)
			P = .60
Any major vascular event	5930 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10



The number of events by allocated treatment are presented for individual trials and subgroups of trials; participants can contribute only once to subtotals and totals of major vascular events. Rate ratios for individual trials or subgroups of trials are indicated by squares and 99% CIs by horizontal lines. Overall totals and their 95% confidence intervals are represented by diamonds. The size of the squares and the diamonds are proportional to the statistical information conveyed.

dose of 1800 mg EPA alone. The daily doses of EPA varied from 226 to 1800 mg/day, and DHA varied from 0 to 1700 mg/day. The mean duration of treatment in individual trials varied from 1.0 year to 6.2 years (weighted mean, 4.4 years).

Of the 77 917 participants, 47 803 (61.4%) were men, and the mean age at entry was 64 years. After accounting for missing data, about two-thirds of participants had a prior history of CHD (31 076/46 767; 66.4%), 13 240 of 47 938 (28%) had prior stroke, and 28 722 of the total 77 917 participants (37%) had prior diabetes. Among the 77 917 participants, there were a total of 12 001 major vascular events (15.4% of 77 917 participants), including 2276 incidents of nonfatal MI (2.9%), 2695 CHD deaths (3.5%), 1713 strokes (2.2%), and 6603 revascularization events (8.5%) during the study duration (eTable in the Supplement). Data were available on the association of treatment by prior use of statin therapy in 7 trials involving 49 522 participants.[8,10-12,14,15]

Associations of Omega-3 Fatty Acid Use With CHD and Major Vascular Events

Figure 1 shows that randomization to receive omega-3 FA supplementation had no significant association with the rate ratios (RRs) for any CHD event (RR, 0.96; 95% CI, 0.90-1.01; *P* = .12) and no significant association with RRs in subgroups of CHD events, including CHD death (RR, 0.93; 99% CI, 0.83-1.03; *P* = .05) and nonfatal myocardial infarction (RR, 0.97; 99% CI, 0.87-1.08; *P* = .40). Likewise, randomization of patients to an omega-3 FA supplementation regimen had no associations with the RRs for major vascular events (RR, 0.97; 95% CI, 0.93-1.01; *P* = .10), stroke (RR, 1.03; 95% CI, 0.93-1.13; *P* = .56), or revascularization events (RR, 0.99; 95% CI, 0.94-1.04; *P* = .61). This meta-analysis also showed no significant heterogeneity between the results of individual trials for nonfatal MI, CHD death, any CHD events, or all major vascular events (**Figure 2**). The association of omega-3 FA supplementation with major vascular events were unaltered after excluding the JELIS trial[14] (odds ratio [OR], 0.98; 95% CI, 0.94-1.02; *P* = .30) (eFigure 3 in the Supplement). Additional sensitivity analyses in 1 trial[12] that compared the results of the Peto method (O−E statistic) with the log-rank method demonstrated that analysis of individual participant and study-level data yielded identical results for association of omega-3 FA supplementation with major vascular events (eFigure 4 in the Supplement).

Associations of Omega-3 Fatty Acid Use With Major Vascular Events in Prespecified Subgroups

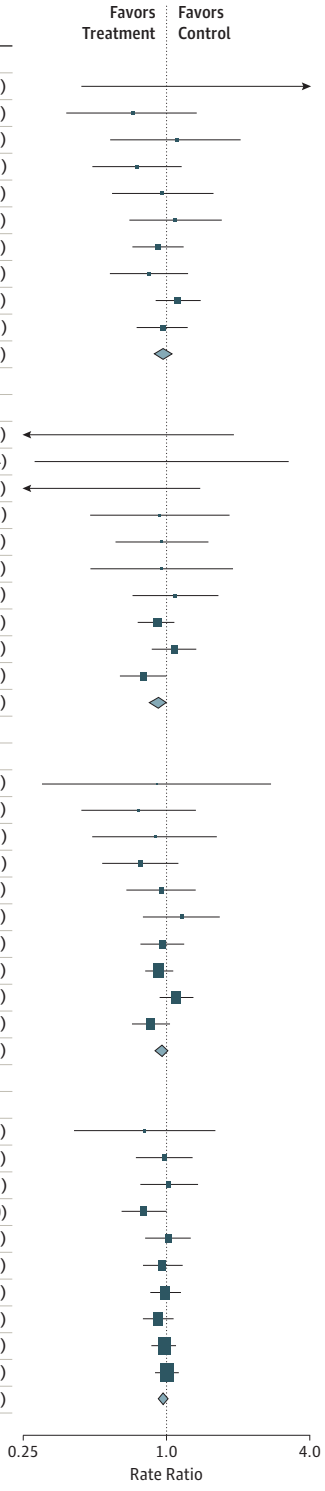
Figure 3 shows that after adjustment for multiple testing, randomization of patients to study arms involving supplementation by omega-3 FAs had no significant association with major vascular events in any of the prespecified subgroups, including those defined by sex, history of CHD, history of diabetes, pretreatment levels of total cholesterol, high-density lipoprotein levels, low-density lipoprotein levels, triglyceride levels, or prior use of statin therapy. However, there was some evidence of heterogeneity in the associations of omega-3 FAs with major vascular events by age (unadjusted *P* = .02) and by history of stroke (*P* = .06), respectively. While it was not possible to assess the associations of treatment with race, the results were unaltered after exclusion of the JELIS trial,[14] which was conducted in a Japanese population only (eFigure 3 in the Supplement).

Associations of Omega-3 Fatty Acid Use With CHD Events by Study Design

Figure 4 demonstrates that randomization of patients to receive omega-3 FAs had no significant association with their experience of nonfatal MI, CHD death, or overall CHD in trials that used either an open-label and blind design. However, there was some evidence of heterogeneity between the results of open-label trials vs blind trials for all participants with CHD (open-

Figure 2. Associations of Omega-3 Fatty Acids With Subtypes of Coronary Heart Disease and Major Vascular Events, by Trial

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Nonfatal myocardial infarction			
DOIT	8 (2.8)	4 (1.4)	1.97 (0.44-8.85)
AREDS-2	30 (1.4)	40 (1.9)	0.72 (0.38-1.33)
SU.FOL.OM3	32 (2.6)	29 (2.3)	1.10 (0.56-2.15)
JELIS	62 (0.7)	83 (0.9)	0.75 (0.49-1.15)
Alpha Omega	56 (2.3)	59 (2.4)	0.96 (0.59-1.56)
OMEGA	74 (3.8)	67 (3.6)	1.08 (0.70-1.69)
R&P	228 (3.7)	248 (4.0)	0.92 (0.72-1.17)
GISSI-HF	92 (2.6)	108 (1.3)	0.84 (0.58-1.22)
ORIGIN	316 (5.0)	284 (4.5)	1.11 (0.90-1.38)
GISSI-P	223 (3.9)	233 (4.1)	0.96 (0.75-1.22)
All	1121 (2.9)	1155 (3.0)	0.97 (0.89-1.05)
			P = .40
Coronary heart disease death			
DOIT	3 (1.1)	8 (2.8)	0.39 (0.08-1.90)
AREDS-2	9 (0.4)	9 (0.4)	0.96 (0.28-3.24)
SU.FOL.OM3	5 (0.4)	13 (1.0)	0.41 (0.12-1.38)
JELIS	29 (0.3)	31 (0.3)	0.93 (0.48-1.82)
Alpha Omega	67 (2.8)	71 (2.9)	0.95 (0.61-1.49)
OMEGA	28 (1.5)	29 (1.5)	0.95 (0.48-1.89)
R&P	82 (1.3)	76 (1.2)	1.08 (0.72-1.64)
GISSI-HF	512 (14.7)	555 (15.9)	0.91 (0.76-1.07)
ORIGIN	319 (5.1)	296 (4.7)	1.08 (0.87-1.33)
GISSI-P	247 (4.4)	306 (5.4)	0.80 (0.64-1.00)
All	1301 (3.3)	1394 (3.6)	0.93 (0.85-1.00)
			P = .05
Any coronary heart disease			
DOIT	11 (3.9)	12 (4.3)	0.91 (0.30-2.73)
AREDS-2	39 (1.8)	49 (2.4)	0.76 (0.44-1.32)
SU.FOL.OM3	37 (3.0)	41 (3.3)	0.90 (0.49-1.62)
JELIS	88 (0.9)	113 (1.2)	0.78 (0.54-1.12)
Alpha Omega	120 (5.0)	128 (5.3)	0.95 (0.68-1.32)
OMEGA	112 (5.8)	96 (5.1)	1.16 (0.80-1.67)
R&P	130 (5.0)	324 (5.2)	0.96 (0.78-1.18)
GISSI-HF	1309 (37.5)	1360 (39.1)	0.93 (0.82-1.06)
ORIGIN	635 (10.1)	580 (9.3)	1.10 (0.94-1.29)
GISSI-P	424 (7.5)	485 (8.6)	0.86 (0.72-1.03)
All	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12
Major vascular events			
DOIT	29 (10.3)	35 (12.5)	0.81 (0.41-1.60)
AREDS-2	213 (9.9)	208 (10.1)	0.98 (0.75-1.28)
SU.FOL.OM3	216 (17.2)	211 (16.9)	1.02 (0.78-1.35)
JELIS	262 (2.8)	324 (3.5)	0.80 (0.65-1.00)
Alpha Omega	332 (13.8)	331 (13.6)	1.02 (0.82-1.26)
OMEGA	534 (27.7)	541 (28.6)	0.96 (0.80-1.16)
R&P	733 (11.7)	745 (11.9)	0.99 (0.86-1.14)
GISSI-HF	783 (22.4)	831 (23.9)	0.92 (0.80-1.07)
ORIGIN	1276 (20.3)	1295 (20.7)	0.98 (0.87-1.09)
GISSI-P	1552 (27.4)	1550 (27.3)	1.00 (0.90-1.12)
All	5930 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10

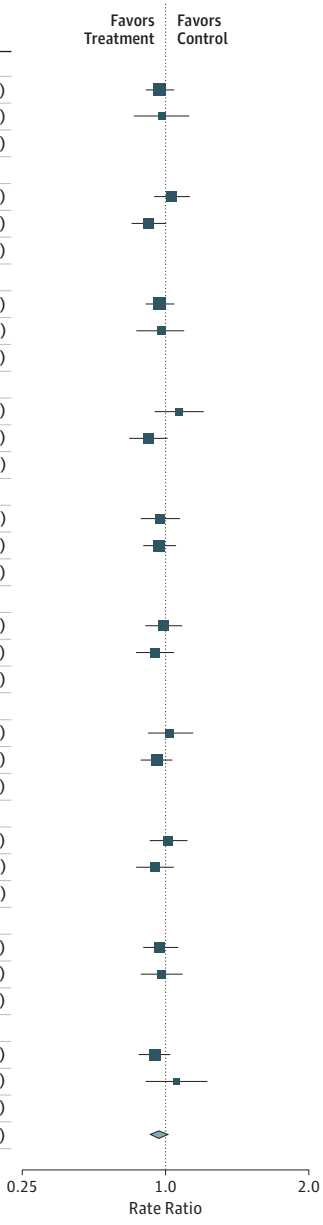


Symbols and conventions as in Figure 1. Study names are AREDS-2, Age-Related Eye Disease Study 2; DOIT, Diet and Omega-3 Intervention Trial; GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Heart Failure; GISSI-P, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione; JELIS, Japan Eicosapentaenoic Acid (EPA) Lipid Intervention Study; OMEGA, Effect of Omega 3-Fatty Acids on the Reduction of Sudden Cardiac Death After Myocardial Infarction; ORIGIN, Outcome Reduction With Initial Glargine Intervention; SU.FOL.OM3, Supplémentation en Folates et Omega-3; R&P, Risk and Prevention Study. Rate ratios for individual trials or subgroups of trials are indicated by squares and the 99% CIs by the horizontal lines. Overall totals and their 95% confidence intervals are represented by diamonds. Arrowheads indicate error bars that extend beyond the area shown. Heterogeneity between all trials (χ^2_9 in all cases) for nonfatal myocardial infarction, coronary heart disease death, any coronary heart disease, and major vascular events were 10.18 (*P* = .34), 12.3 (*P* = .20), 12.92 (*P* = .17), and 7.68 (*P* = .57), respectively.

label trials: RR, 0.85; 99% CI, 0.72-0.99; *P* = .01; blinded trials: RR, 0.99; 99% CI, 0.91-1.07; *P* = .69; heterogeneity *P* = 0.03), but not for either fatal CHD or nonfatal MI, respectively. Overall, the results of this meta-analysis demonstrated no significant association of supplementation with omega-3 FAs for a mean duration of 4.4 years with the risk of fatal CHD, nonfatal MI, any CHD, or any major vascular events in the full study population and in all relevant subgroups.

Figure 3. Associations of Omega-3 Fatty Acids With Major Vascular Events, in Prespecified Subgroups

Source	No. of Events (%) Treatment (n = 39 017)	Control (n = 38 900)	Rate Ratios (CI)
Sex			
Male	3989 (23.2)	4040 (23.7)	0.97 (0.91-1.04)
Female	946 (15.1)	962 (15.5)	0.98 (0.86-1.12)
Missing	995 (6.4)	1069 (6.9)	0.93 (0.82-1.04)
Age, y			
<65	2528 (21.8)	2432 (21.3)	1.03 (0.95-1.12)
≥65	2407 (20.5)	2570 (22.0)	0.92 (0.85-1.00)
Missing	995 (6.4)	1069 (6.9)	0.93 (0.82-1.04)
Prior coronary heart disease			
Yes	3499 (22.5)	3550 (22.9)	0.97 (0.91-1.04)
No	1435 (18.5)	1448 (19.0)	0.98 (0.87-1.09)
Missing	996 (6.4)	1073 (6.9)	0.92 (0.82-1.04)
Prior stroke			
Yes	1332 (21.1)	1262 (20.0)	1.07 (0.95-1.20)
No	2046 (18.3)	2185 (19.6)	0.92 (0.84-1.01)
Missing	2552 (12.0)	2624 (12.3)	0.97 (0.89-1.05)
Prior diabetes			
Yes	2087 (22.8)	2111 (23.3)	0.97 (0.89-1.07)
No	2846 (20.1)	2889 (20.5)	0.97 (0.90-1.05)
Missing	997 (6.4)	1071 (6.9)	0.93 (0.82-1.04)
Total cholesterol			
<193.1	2326 (21.5)	2315 (21.5)	0.99 (0.91-1.08)
≥193.1	2346 (22.8)	2441 (23.7)	0.95 (0.87-1.04)
Missing	1258 (7.1)	1315 (7.4)	0.94 (0.84-1.05)
High-density lipoprotein cholesterol			
<38.6	1532 (25.4)	1512 (24.8)	1.02 (0.92-1.14)
≥38.6	2961 (20.7)	3041 (21.5)	0.96 (0.89-1.03)
Missing	1437 (7.8)	1518 (8.2)	0.94 (0.85-1.04)
Low-density lipoprotein cholesterol			
<115.8	2096 (21.0)	2051 (20.6)	1.01 (0.93-1.11)
≥115.8	2295 (23.2)	2385 (24.2)	0.95 (0.87-1.04)
Missing	1539 (8.1)	1635 (8.6)	0.93 (0.84-1.03)
Triglycerides			
≤150.4	2626 (21.3)	2638 (21.7)	0.97 (0.90-1.06)
>150.4	1998 (23.4)	2061 (23.7)	0.98 (0.89-1.08)
Missing	1306 (7.3)	1372 (7.7)	0.94 (0.84-1.04)
Prior statin use			
Yes	2824 (11.4)	2959 (11.9)	0.95 (0.88-1.02)
No	742 (15.4)	696 (14.9)	1.05 (0.91-1.22)
Missing	2364 (25.0)	2416 (25.6)	0.97 (0.89-1.06)
All	5930 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10



Symbols and conventions as in Figure 1. Total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were measured in mg/dL (to convert cholesterol to mmol/L, multiply by 0.0259; triglycerides, multiply by 0.0113). Heterogeneity between all trials (χ^2_1 in all cases) was 0.04 (P = .84) for sex, 5.59 (P = .02) for age, 0.0 (P = .96) for prior coronary heart disease, 7.03 (P = .01) for prior stroke, 0.0 (P > .99) for prior diabetes, 0.87 (P = .35) for total cholesterol, 1.56 (P = .21) for high-density lipoprotein cholesterol, 1.8 (P = .18) for low-density lipoprotein cholesterol, 0.02 (P = .89) for triglycerides, and 2.55 (P = .11) for prior statin use.

Associations of Omega-3 Fatty Acid Use With All-Cause Mortality

Randomization to omega-3 FA intervention had no significant association with RRs of all-cause mortality (RR, 0.96; 95% CI, 0.92-1.01; P = .16). Further information is presented in eFigure 5 in the Supplement.

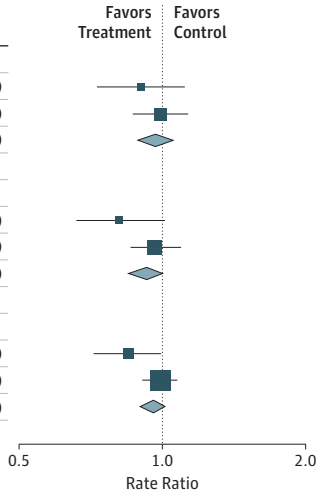
Discussion

This meta-analysis of 10 randomized clinical trials, involving 77 917 participants, demonstrated that randomization to trial arms with omega-3 FA supplementation for a mean of 4.4 years had no significant effect on either of fatal CHD, nonfatal MI, stroke, revascularization events, or any major vascular events. Importantly, this meta-analysis also demonstrated no significant effect on major vascular events in any particular subgroups, including prior vascular disease, diabetes, lipid levels, or statin use. Likewise, the present meta-analysis showed no significant association of omega-3 FA supplementation with all-cause mortality or cancer (data not shown). Moreover, the overall results were unaltered after exclusion of the JELIS trial,[14] which tested the effects of EPA alone rather than EPA and DHA combined.

The chief strength of this study was the availability of study-level data extracted by the trial principal investigators for all prespecified outcomes in this meta-analysis (with the exception of the JELIS trial,[14] in which the published data

Figure 4. Associations of Omega-3 Fatty Acids With Fatal and Nonfatal Vascular Events, by Trial Design

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Nonfatal myocardial infarction			
Open	285 (1.9)	316 (2.1)	0.90 (0.73-1.11)
Blind	836 (3.5)	839 (3.5)	0.99 (0.87-1.13)
All	1121 (2.9)	1155 (3.0)	0.97 (0.89-1.05)
			P = .40
Coronary heart disease death			
Open	276 (1.8)	337 (2.2)	0.81 (0.66-1.01)
Blind	1025 (4.3)	1057 (4.4)	0.96 (0.86-1.09)
All	1301 (3.3)	1394 (3.6)	0.93 (0.85-1.00)
			P = .05
Any coronary heart disease			
Open	512 (3.4)	598 (4.0)	0.85 (0.72-0.99)
Blind	2573 (10.7)	2590 (10.8)	0.99 (0.91-1.07)
All	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12



Symbols and conventions as in Figure 1. Heterogeneity between trial designs (χ^2_1 in all cases) was 1.05 (*P* = .31) for nonfatal myocardial infarction, 3.26 (*P* = .07) for coronary heart disease death, and 4.81 (*P* = .03) for any coronary heart disease.

were used). The inclusion criteria and vascular disease outcomes differed from previous meta-analyses of the published results.[16-18] The present meta-analysis had a low risk of selection bias or confounding because it did not include trials testing the effects of dietary advice to eat fish nor trials that were either too small or insufficient in treatment duration. In contrast with previous meta-analyses, the present meta-analysis also examined effects of supplementation with omega-3 FA supplementation in prespecified subgroups of major vascular events by history of disease, history of diabetes, lipid levels, or statin use.

The reasons for the discrepant results of the previous trials of omega-3 FA supplementation on fatal and nonfatal CHD events are unclear. In contrast with the null findings for most trials, the GISSI-Prevenzione trial[6] reported a 14% reduction in major vascular events, chiefly owing to an 11% reduction in cardiac deaths. But the JELIS trial reported a 19% (95% CI, 5%-31%) reduction in major CHD events (albeit based on only 586 events), chiefly owing to a reduction in nonfatal CHD events.[14] It is unclear whether differences in inclusion criteria for prior diseases, concomitant use of statins, or other secondary prevention treatments may explain some of the conflicting results of individual trials.

For example, previous reports had suggested that the effects of omega-3 FA use may vary by patients' prior use of statin medications.[21,22] The Alpha Omega trial reported that use of low-dose omega-3 FAs reduced the risk of major vascular events in patients with prior MI who were not treated with statin medications.[22] However, the present meta-analysis demonstrated no heterogeneity in the effects of omega-3 FA supplementation on CHD death or nonfatal MI between the individual trials and reported no differences in the effects of omega-3 FAs on major vascular events by subgroups of those with or without prior cardiovascular disease or diabetes; those with lipid levels less than or greater than specified cutoff points; or those who had histories of statin therapy. The results of the present meta-analysis were also unaltered by the exclusion of the JELIS trial,[14] in which all participants were also treated with statin medications.

The present meta-analysis reported weak evidence of heterogeneity between the results of open-label vs blind trials for any CHD. This may reflect reporting bias, chance, or greater compliance in the open-label trials than in the blinded trials.

Previous meta-analyses of omega-3 FA trials,[16-18] which were limited by being incomplete, including trials of dietary advice to increase fish consumption,[16,17] or failure to distinguish the effects on a wide range of subtypes of CVD.[16-18,23,24] In contrast, the present meta-analysis demonstrated that omega-3 FA supplementation had no significant effect on fatal CHD or any other CVD subtypes. Moreover, the conclusions of the present meta-analysis are consistent with those of a 2016 report[24] for the US Agency for Healthcare Research and Quality that also involved study-level data from the same 10 large trials for prevention of major vascular events, and concluded that omega-3 FA supplementation had no association with the risk of major vascular events, all-cause mortality, sudden cardiac death, or revascularization. In contrast with this report, the present article was able to assess effects on a wide range of subtypes of CVD and on major vascular events in all relevant subgroups.[24]

Limitations

This meta-analysis had several limitations. The protocol did not prespecify assessment of the effects of treatment by smoking status or by site-specific cancer incidence. An additional limitation of this meta-analysis involved the use of aggregated study-level data rather than individual-level data. A meta-analysis of individual participant data may have a greater chance of detecting effects of omega-3 FA supplements on subtypes of fatal CHD events (ie, sudden death or ventricular arrhythmias) in a wider range of subgroups. However, the overall null results of the present meta-analysis, which assesses effects on a wide range of prespecified CVD subtypes, provides little encouragement for such an approach. In addition, sensitivity analyses

using data from 1 trial[12] that also provided data on all individual participants indicated identical effect estimates and 99% CI for analyses using both O-E and log-rank methods.

The 95% CI in the present meta-analysis of 10 trials, involving 77 917 high-risk individuals, 12 001 major vascular events, and 6273 CHD events, cannot exclude a 7% lower risk of major vascular events and a 10% lower risk of CHD associated with omega-3 FA supplements. Several ongoing large randomized trials involving a total of 54 354 additional participants (A Study of Cardiovascular Events in Diabetes [ASCEND],[25] n = 15 480; VITamin D and OmegA-3 TriaL [VITAL],[26] n = 25 874; STatin Residual risk reduction with EpaNova in hiGh CV risk patienTs with Hypertriglyceridemia [STRENGTH],[27] n = 13 000 and Reduction of Cardiovascular Events With EPA–Intervention Trial [REDUCE-IT], n = 8000) will provide additional evidence about the associations of omega-3 FA supplementation with the risk of major vascular events, any CHD, and subtypes of fatal and nonfatal CHD. Importantly, the STRENGTH[27] and REDUCE-IT trials will test the effects on major vascular events of much higher doses of omega-3 FAs (3-4 g/d), which will lower plasma levels of triglycerides.

Conclusions

The 2016 European Society of Cardiology and European Atherosclerosis Society guidelines for prevention of cardiovascular disease[28] indicated that it is debatable whether omega-3 FAs may exert a protective effect, and the 2016 guidelines on the management of dyslipidaemia[29] indicated that more evidence on the efficacy of omega-3 FA supplements for prevention of clinical outcomes is needed to justify their prescription. In contrast, the American Heart Association recommended[30] that the use of omega-3 FAs for prevention of CHD is probably justified in individuals with prior CHD and those with heart failure and reduced ejection fractions. However, the results of the present meta-analysis provide no support for the recommendations to use approximately 1 g/d of omega-3 FAs in individuals with a history of CHD for the prevention of fatal CHD, nonfatal MI, or any other vascular events. The results of the ongoing trials are needed to assess if higher doses of omega-3 FAs (3-4 g/d) may have significant effects on risk of major vascular events.

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The Use of Multivitamin/Multimineral Supplements: A Modified Delphi Consensus Panel Report

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ABSTRACT

Purpose: Evidence supporting the use of dietary supplements, in particular, multivitamin/multimineral supplements (MVMS), has been mixed, complicating the ability of health care professionals to recommend their use. To clarify the role that MVMS can play in supporting human health, a series of consensus statements was developed based on expert opinion.

Methods: A panel of 14 international experts in nutritional science and health care was convened to develop consensus statements related to using MVMS in supporting optimal human health. The modified Delphi process included 2 rounds of remote voting and a final round of voting at a roundtable meeting where evidence summaries were presented and discussed. The level of agreement with each of 9 statements was rated on a 5-point Likert scale: agree strongly; agree with reservation; undecided; disagree; or disagree strongly. Consensus was predefined as ≥80% of the panel agreeing strongly or agreeing with reservation to a given statement.

Findings: Consensus was reached for all statements. The panel determined that MVMS can broadly improve micronutrient intakes when they contain at least the micronutrients that are consumed insufficiently or have limited bioavailability within a specified population. MVMS formulations may also be individualized according to age, sex, life cycle, and/or other selected characteristics. There are specific biological processes and health outcomes associated with deficient, inadequate, and adequate micronutrient levels. Adequate intake is necessary for normal biological functioning required for good health; in some instances, higher

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than recommended micronutrient intakes have the potential to provide additional health benefits. Meeting daily intakes established by dietary reference values should be an explicit public health goal for individuals and populations. Use of MVMS is one approach to ensure that adequate micronutrient needs are met in support of biological functions necessary to maintain health. Long-term use of MVMS not exceeding the upper limit of recommended intakes has been determined to be safe in healthy adults. There is insufficient evidence to indicate that MVMS are effective for the primary prevention of chronic medical conditions, including cardiovascular disease and cancer. However, for certain otherwise healthy subpopulations (eg, pregnant women, older adults) and some individuals with existing medical conditions who experience inadequacies in micronutrient intake, addressing inadequacies by using MVMS can provide health benefits.

Implications: This consensus panel has described key issues related to the use of MVMS among individuals at risk of or presenting with inadequacies in micronutrient intake or biomarker status. (*Clin Ther*. 2018;40:640–657)

Key words: adverse effects, Delphi consensus, dietary supplements, health benefits, multivitamin/multimineral supplements, nutrition.

INTRODUCTION

Dietary supplements and, in particular, multivitamin/multimineral supplements (MVMS), are widely used[1]; recent data from the United States suggest that the use of MVMS is declining, however.[1,2] No guidelines currently exist for recommending the use of MVMS, and nutritional education and training among health care professionals (HCPs), including physicians, nurses, and pharmacists, are limited.[3–7] Thus, little direction is available for HCPs to guide patients in this area. Results from randomized controlled trials (RCTs) conducted with MVMS provide conflicting evidence about their potential benefits in preventing/treating chronic medical conditions (CMCs), leading some to question their value, particularly in higher income countries.[8,9] Nonetheless, there is ample evidence from national dietary intake surveys reporting deficiencies and inadequacies in micronutrient intake and/or status, and correcting these deficiencies can have health benefits.[10,11] To address this conundrum, an international panel of experts in the areas of nutritional science and health care was convened to develop consensus statements that discuss issues regarding MVMS use.

An essential component in discussing the role of dietary supplements involves defining recommended intakes for maintaining good health. Vitamin and mineral requirements are defined as the intake needed to meet a specified indicator of adequacy for each nutrient.[12] The terms commonly used to describe reference intakes are defined in Figure 1.[13] However, it is important to appreciate that dietary reference values can vary among countries or regions based on different criteria and/or approaches to reach consensus.

MATERIALS AND METHODS

An international group of 14 experts in nutritional science and health care was convened to develop a series of consensus statements that present guidelines for using MVMS. To ensure that the panel was composed of a heterogeneous group of experts in the specialty area and to provide global representation that would allow for regional variations to be accounted for in the statements, the consensus panel's co-chairs (J.B.B. and H.C.) identified a select number of participants based on their expertise and geographic location beginning in November 2016. After initially contacting the co-chairs, the sponsor (Pfizer Consumer Healthcare, Madison, New Jersey) had no involvement in conducting the consensus panel or preparing the present article.

Once participants were identified, the co-chairs developed a set of initial questions related to using MVMS. Each assigned panel member, as selected by the co-chairs, began searching the literature and identifying information sources to address these questions. Where applicable, the methods for conducting literature reviews are described in the corresponding evidence summaries. After reviewing the literature, panelists selected as Statement Leads developed initial drafts of each statement, which were shared with team members chosen by the co-chairs to assist in this effort. The initial 9 statements were then circulated to the entire panel for a first round of remote consensus voting. The voting followed a modified Delphi process,[14,15] in which the level of agreement

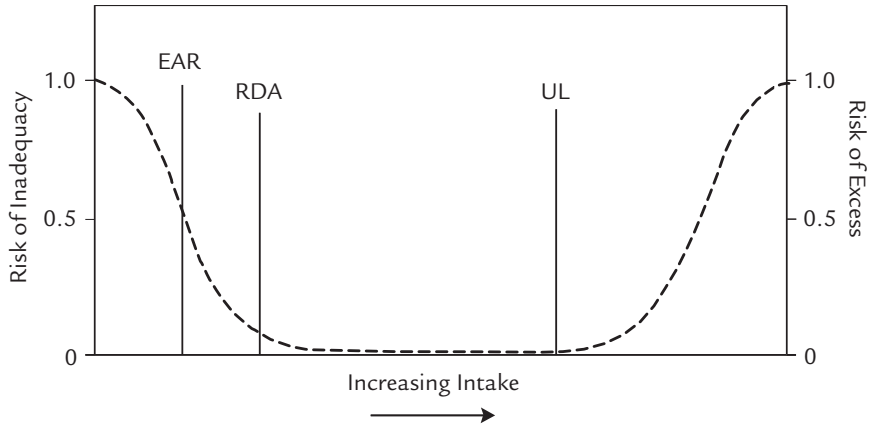
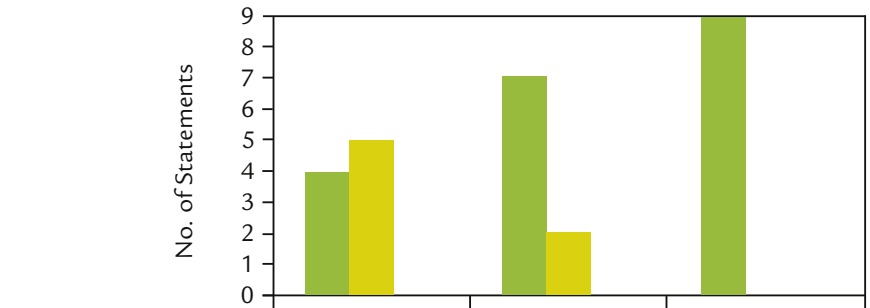


Figure 1. Definitions of terms used to describe components of Dietary Reference Intakes.[11] This figure shows that the Estimated Average Requirement (EAR) is the intake at which the risk of inadequacy is 0.5 (50%) for an individual. The Recommended Dietary Allowance (RDA) is the intake at which the risk of inadequacy is very small, only 0.02 to 0.03 (2% to 3%). The Adequate Intake (AI) does not bear a consistent relationship to the EAR or the RDA, because it is set without the estimate of the requirement. As a result, the AI is not included in this figure. At intakes between the RDA and the Tolerable Upper Intake Level (UL), the risks of inadequacy and excess are both close to 0. At intakes above the UL, the risk of adverse effects may increase. AI is the recommended average level of daily nutrient intake based on observed or experimentally determined approximations of intake by a group (or groups) of apparently healthy people that are assumed to be adequate; it is used when an RDA cannot be determined. Mean usual intake at or above this level has a low probability of inadequacy among individuals or groups. When the AI for a nutrient is not based on mean intakes of healthy populations, the assessment of adequacy is made with less confidence. EAR is the average daily nutrient intake level estimated to meet the requirement of one half the healthy individuals in a particular life stage and sex group; it is used to examine the probability that usual intake is inadequate in an individual or to estimate the prevalence of inadequate intakes within a group. Provided certain assumptions are met, the prevalence of inadequate intakes in a group can be estimated as the percentage of the group's usual intake distribution that falls below the EAR. The RDA is the average daily nutrient intake level that is sufficient to meet the nutrient requirement of nearly all (97%–98%) healthy individuals in a particular life stage and sex group. For nutrients with normal requirement distributions, the RDA is calculated from the EAR by adding 2 SDs of the requirement distribution to the EAR. Usual intake at or above the RDA has a low probability of inadequacy for an individual; it is not to be used to assess intakes of groups. The UL is the highest average daily nutrient intake level likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the potential risk of adverse effects increases. Usual intake above this level may place an individual at risk of adverse effects from excessive nutrient intake; it is used to estimate the percentage of the population at potential risk of adverse effects from excessive nutrient intake. Adapted with permission of National Academies Press from *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington, DC: National Academies Press; 2000; permission conveyed through Copyright Clearance Center, Inc.

was rated on a 5-point Likert scale: agree strongly (A+); agree with reservation (A); undecided (U); disagree (D); or disagree strongly (D+). Consensus was predefined as ≥80% of the panel rating a given statement A+ or A.

After the Statement Leads and their team members revised the statements, a subsequent round of remote voting was conducted; the final round of voting was conducted at an in-person meeting of all panelists in Philadelphia, Pennsylvania, in June 2017, where the Statement Leads presented evidence summaries used to support their statements. The live meeting, which was moderated by the co-chairs, was included to allow the panel members to openly discuss the statements. The



(N = 14 participants)	Round 1	Round 2	Round 3
≥80% agreement	4	7	9
≥50% to 79% agreement	5	2	0
<50% agreement	0	0	0

Figure 2. Evolution of consensus voting over 3 rounds.

meeting was followed by the final round of anonymous voting using an automated response system.[16] The co-chairs decided to forego rating the quality of evidence and strength of recommendations because these criteria would not be applicable to most statements; where applicable, the quality of evidence is informally described. A summary of the consensus voting is presented in Figure 2.

RESULTS

Consensus was reached for all statements. Upon completion of the in-person meeting, a confidential poll was conducted and panel members agreed that no commercial bias was evident in the process before or during the meeting. Also, all participants provided adequate input on all statement ratings and recommendations, which are described individually in the following sections.

1. For the purpose of broad-spectrum micronutrient supplementation for a general population, MVMS should contain at least the micronutrients that are commonly underconsumed relative to their recommended intakes within that country/region. Most of these vitamins and nutritionally essential minerals should be present in amounts approximating recommended intakes. Within this context, MVMS may be safely formulated for large subgroups according to age, sex, and/or life-cycle–specific micronutrient needs.

A+: 64%; **A:** 36%

There is currently neither consensus nor published criteria quantifying the doses of micronutrients that should be included in MVMS. However, several definitions of MVMS have been proposed. For example, the US National Institutes of Health defines MVMS as: "any supplement containing 3 or more vitamins and minerals but no herbs, hormones, or drugs, with each component at a dose less than the Tolerable Upper Intake Level (UL) determined by the Food and Nutrition Board—the maximum daily intake likely to pose no risk for adverse health effects."[17] The National Institutes of Health classifies MVMS into subgroups as: (1) "once daily," which contain most or all vitamins and essential minerals at levels approximating the Recommended Dietary Allowance (RDA) or Adequate Intake (AI); (2) special formulations designed for specific subpopulations, and packs containing multiple individual supplements; and (3) "specialized" formulations that might contain vitamins and minerals at levels substantially above the RDA and sometimes the UL.[18] In contrast, others have defined MVMS as dietary supplements providing ≥100% of the RDA or AI for ≥9 to 10 vitamins and nutritionally essential minerals.[2,10,11] Currently available MVMS do not generally contain the RDA or AI for calcium, choline, magnesium, potassium, vitamin K, phosphorus, and others.

Evidence from international databases, review articles, and national population-based surveys indicates that micronutrient intake deficiencies/inadequacies

(especially for vitamin A, iron, iodine, folate, and other B vitamins, vitamins D and E, magnesium, and calcium) occur on a global scale and are mirrored by low intakes from foods and supplements.[10,19–27] Despite the absence of established regulatory and scientific definitions for MVMS, there is agreement that these products should minimally contain the vitamins and minerals that are commonly underconsumed relative to their RDA or AI within a country/region in amounts below the UL. In addition, MVMS may be safely formulated for specific subgroups according to age, sex, and/or life-cycle–specific micronutrient needs. The decision by HCPs to recommend MVMS can be individualized based on a person's diet and risk for nutrient deficiencies/inadequacies.[28] Importantly, MVMS are considered to be supplements, not substitutes, for a balanced diet. Indeed, MVMS are generally perceived by consumers as an "insurance policy" to help achieve adequate micronutrient intake. MVMS that maintain intake at or below the RDA or AI are unlikely to result in excess intake, even when including the contribution of diet and fortification, although use of additional supplements might increase the risk of exceeding the UL.[11,29–31]

2. Several factors are associated with deficient, inadequate, or adequate micronutrient intake: biological functions; cellular, metabolic, or physiological states; and health outcomes. For some micronutrients, higher intakes might provide added health benefits.

A+: 50%; **A:** 50%

Micronutrients have distinct biological functions, including serving as essential co-factors to many enzymes and as structural elements of biological macromolecules (eg, B vitamins and DNA synthesis), involvement in one-carbon metabolism, and acting as hormones and antioxidants.[32–35] These biological functions are essential to metabolic functioning, growth and development, and many cellular and organ system functions. In most cases, RDAs and AIs are based on specific biological or physiological indicators for each micronutrient to prevent deficiency diseases/syndromes. For example, Dietary Reference Intakes (DRIs) of vitamin D and calcium are intended to promote bone mineral density in adolescents and reduce loss with aging, and those for folate are linked to the prevention of megaloblastic anemia and neural tube defects (NTDs).[33,35]

When micronutrient intakes are inadequate, suboptimal cellular/physiological functions can occur in advance of developing a classic symptomatic deficiency condition.[33] For example, inadequate vitamin A stores are associated with immunodeficiency. Several other micronutrients in addition to vitamin A play important roles in innate and adaptive immunity (eg, vitamin D), and inadequacies impair normal immune function, which may increase the risk of infectious diseases and cancer.[33,35] Potassium inadequacies are associated with hypertension and increased risk of myocardial infarction and stroke.[33,36] Inadequacies in intakes of folate and vitamin C are associated with biomarkers indicating an increased risk for hyperhomocysteinemia, chromosome breakage, chronic inflammation, and oxidative damage.[33,37]

Although micronutrient inadequacies are associated with risk of deficiency and impaired biological functioning, more research is necessary to establish clear dose–response relationships between biological functional status and health outcomes, taking into consideration interindividual and regional differences in diet, lifestyle, environment, and genetic variants. This effort could also clarify apparent increases in function beyond those associated with deficiencies and inadequacies, as suggested by studies of higher or "optimal" doses of vitamins C and D showing apparent improvements in physiological functioning and risk reduction for age-related chronic diseases.[38–43]

3. Achieving micronutrient intake levels on a population-wide and individual basis that are consistent with established reference values should be an explicit public health goal.

A+: 64%; **A:** 36%

Human health requires complete and balanced nutritional intake; however, inadequacies in vitamin and mineral intakes have been widely described, not only in impoverished and undernourished populations[44,45] but also in developed countries with apparently sufficient resources.[46] Micronutrients are available via the diet, food fortification, supplementation, or a combination of these approaches,[47] with dietary micronutrient intake being influenced by numerous factors that change over time.[44]

Global recommendations for micronutrient intake include DRIs, which are developed by the US National Academy of Medicine[48] and Health Canada,[12] and Dietary Reference Values developed by the UK

Scientific Advisory Committee on Nutrition[49] and the European Union Food Safety Authority,[50] and Nutrient Reference Values developed for Australia and New Zealand.[51] Technical support documents provide the scientific evidence for recommendations of each micronutrient and reference values developed to direct public policy decisions.[52,53] The World Health Organization[44] and the Food and Agriculture Organization of the United Nations[54] recognize the critical role that governments play in setting national policies to promote adequate nutrient intake and protect public health. Support for nutrition guidelines in public policy is also expressed in the United Nations Sustainable Development Goals to improve nutrition and protect good health and well-being.[55]

Setting public health goals to achieve recommended micronutrient intakes at individual and population levels presents challenges with implementation and monitoring. In setting public policy, these efforts should appreciate existing knowledge gaps for some micronutrient recommendations and recognize the diversity of individuals and populations and their respective dietary requirements. Consistent with current practices, meeting micronutrient needs for individuals and populations often requires a combination of improving dietary patterns, fortifying staple foods, and supplementing the diet with products such as MVMS. Notably, in some regional programs, focus on ensuring adequate energy intakes for growth and development ignores the risk of "hidden hunger," which results from inadequate micronutrient intakes from low-quality diets and adversely affects global health.[56]

4. Using a daily MVMS is one way to help provide the recommended intake levels of many micronutrients that are necessary for maintaining health through supporting the function of specific metabolic pathways, cells, organs, or other physiological systems.

A+: 43%; **A:** 57%

Due to the difficulty in establishing the effect of MVMS ingredients generally on a given health outcome, to investigate the role of supplements in influencing metabolic and physiological systems, evidence was explored via selected illustrations of priority areas within public health nutrition. These areas included: (1) folate in early life; (2) B vitamins and cognitive health in aging; and (3) vitamin D and health.

The evidence linking inadequate folate status with NTDs is conclusive, and other effects of this B vitamin (and metabolically interrelated B vitamins) throughout the life cycle are becoming evident.[57] Achieving sufficient folate status and biomarkers thereof is challenging because natural food folates are inherently unstable, have limited bioavailability, and can undergo significant losses before ingestion.[58] In contrast, folic acid (ie, the vitamin form found in supplements and fortified food) can overcome these limitations, as it is stable and highly bioavailable. Therefore, improved folate biomarker status is more easily achieved by consuming the vitamin through supplements and fortified food compared with equivalent intakes of folate from natural food sources.[59,60] In the absence of folic acid fortification or supplementation, the average diet does not achieve adequate folate status. Thus, in regions without mandatory folic acid fortification (eg, European Union countries), poor compliance with recommendations to promote folic acid supplements as a policy to prevent NTDs is reflected in evidence showing no change in the prevalence of NTDs over the 20 years since the Medical Research Council Vitamin Study demonstrated that folic acid prevents recurrence of NTDs.[61–63] Furthermore, using folic acid–containing MVMS is proven to reduce the incidence of first occurrence of NTDs[64] and is recommended globally before and in early pregnancy.[62,65]

Beyond NTDs, supplements containing folate-related B vitamins (ie, B_{12}, B_6, riboflavin) may have additional benefits, as they are required for normal folate recycling within one-carbon metabolism. Important gene–nutrient interactions are also recognized in these metabolic pathways, and recent research suggests their impacts on health, such as a novel interaction of riboflavin with the MTHFR gene that affects blood pressure regulation.[66]

Nutritional approaches to slow the progression of age-related cognitive decline are of increasing public health interest due to the growing proportion of elderly people developing these symptoms around the world. Although certain dietary patterns (eg, Mediterranean diet) and specific nutrients (eg, *n*-3 polyunsaturated fatty acids and polyphenols) seem to be associated with cognitive health in aging,[67–69] the

totality of evidence from observational studies and RCTs seems strongest in supporting roles for folate, vitamin B_{12}, and vitamin B_6 through mechanisms related to B vitamin–dependent one-carbon metabolism, a network of pathways essential for healthy cellular functioning, including in the brain.[34,70–72] Recent evidence shows that elderly people with lower B vitamin intake and status have higher rates of cognitive decline.[73] However, additional research is needed to demonstrate that intervention with B vitamin supplements can significantly affect cognitive functioning in older adults.[74]

Vitamin D supplementation can reduce the risk of deficiency (serum 25[OH]D <30 nmol/L) and insufficiency (30–50 nmol/L), which are prevalent globally.[75,76] Vitamin D is essential for bone and muscle function and immune regulation. The preventive effect of vitamin D in osteoporosis is well established[77]; however, the evidence in other areas, although provocative, is less clear. Promising observational evidence supports the role of vitamin D in cognitive health in older adults, but RCTs to date have not confirmed this effect.[78] Recent evidence from a 4-year randomized trial of vitamin D plus calcium supplementation in postmenopausal women did not reduce the incidence of cancer.[79] The results of 3 large RCTs from the United States, Australia, and Finland investigating vitamin D supplementation in relation to cardiovascular disease (CVD) and cancer are expected in the coming years.[80–82]

5. On a population basis, use of daily MVMS reduces the prevalence of inadequate intakes of the micronutrients they contain.

A+: 86%; **A:** 14%

A MEDLINE search was conducted (1946 to May 2017) to obtain evidence regarding the impact of supplementation on nutrient inadequacies, using the following terms: "nutrition surveys" or "diet surveys" or "nutrient adequacy" combined with "dietary supplements" or "multivitamin supplements." Among the 634 citations retrieved, 13 reported intakes of multiple micronutrients by supplement use in national population-based samples.

Surveys conducted in the United States, Canada, Korea, Germany, and Mexico were identified; in several countries, multiple publications were available.[10,11,29–31,83–91] These studies included adults (n = 10) and/or children (n = 7), and most compared total nutrient intakes of supplement users and nonusers. These surveys consistently revealed that supplement users have higher micronutrient intakes and/or lower prevalence of inadequacies or intakes below recommended levels.

Two studies analyzing US National Health and Nutrition Examination Survey datasets specifically assessed the population prevalence of nutrient inadequacies with and without MVMS. Wallace et al[10] found that MVMS use (≥1 day per month), compared with nonuse, significantly reduced the prevalence of inadequate intakes (assessed as nonoverlapping 95% confidence intervals [CIs]) of vitamins A, C, D, and E; calcium; and magnesium. For other micronutrients (eg, thiamine, riboflavin), the prevalence of inadequacy was lower with MVMS use but not statistically significant, in part because of the very low prevalence of inadequacies regardless of supplementation. Blumberg et al[11] found that in relation to intake from food alone, MVMS use at any frequency was associated with a lower prevalence of inadequacies ($P < 0.01$) for 15 of the 17 micronutrients examined. Significant ($P < 0.01$) increases in the prevalence of intakes exceeding the UL for 7 micronutrients were observed, but the prevalence was ≤4% for any micronutrient. Except for calcium, magnesium, and vitamin D, the most frequent category of MVMS use (≥21 days per month) virtually eliminated inadequacies of the nutrients examined. Furthermore, MVMS use was associated with significantly lower odds ratios (ORs) of deficiency for all the examined nutrient biomarkers except for iron.

6. Based on current knowledge, the long-term use of MVMS with an amount not exceeding the UL is safe in healthy adults.

A+: 71%; **A:** 29%

The safety of micronutrients is dependent on their intakes falling between recommended levels and ULs. In an analysis of data from the US National Health and Nutrition Examination Survey (N = 16,444), MVMS reduced the percentage of the population with nutrient intakes below the Estimated Average Requirement but did not cause excess intake.[10] In users of MVMS, the prevalence of those exceeding the UL was 1.7% for retinol and 2.5% for folic acid. Indeed, even if fortified food and beverages are considered, it seems unlikely that intakes will exceed the UL in the

long term. For determining safety, both the amount supplied and the length of use within different age groups should be determined.

Few studies exist that document long-term use and specifically evaluate adverse events (AEs). A review by Simpson et al[92] reported 6 studies of MVMS that used biological safety data from children and adults and reported no clinically meaningful AEs or abnormal blood tests related to toxicity. AE data from 157 children and adults revealed only minor, transitory reports of headache and nausea. One study that directly compared the safety of supplements and conventional psychiatric medications found no clinically meaningful abnormal laboratory values among its 88 pediatric and adult subjects. The supplement group experienced significantly fewer AEs ($P \leq 0.026$) and less weight gain ($P < 0.0001$). In the Physicians' Health Study (PHS) II, the only RCT that has addressed the safety of long-term MVMS use versus placebo, no significant differences were found on gastrointestinal symptoms (eg, peptic ulcer, constipation, diarrhea, gastritis, nausea), fatigue, drowsiness, skin discoloration, or migraine.[93] A systematic review of RCTs by Biesalski and Tinz[94] addressed safety of MVMS; 9 studies evaluated use of MVMS in pregnant women and healthy adults, and 6 studies explicitly assessed AEs in the elderly. Only minor AEs (eg, unspecific gastrointestinal symptoms) were reported, and there were no significant differences between groups. Based on current knowledge, the long-term use of MVMS (>10 years) with doses not exceeding ULs seems to be safe.

7. The evidence that long-term use of MVMS contributes to a reduction in the risk of some chronic diseases is insufficient to support the use of MVMS in the primary prevention of these diseases.

A+: 64%; **A:** 29%; **U:** 7%

In the 21st century, the global burden of disease and associated mortality will continue to be driven primarily by CMCs.[95] The World Health Organization's Global Action Plan for the Prevention and Control of Noncommunicable Diseases proposes policies to increase consumption of fresh fruits and vegetables and reduce energy-rich, micronutrient-poor foods.[96] Evidence from basic research and observational studies indicates that dietary factors, including micronutrients, are key components for preventing the development of CMCs, including some forms of cancer.[97]

In observational studies, the relationship between the total intake of micronutrients at dietary levels and the incidence of CMCs (eg, cancer, CVD, age-related eye diseases) has been shown primarily to be null, and in some instances, positive and even negative associations were reported.[98] In a meta-analysis of 13 cohort studies conducted by Park et al,[99] an aggregate analysis of individuals enrolled in 10 studies using MVMS for 7 to 20 years found a decreased risk of colon cancer (relative risk [RR], 0.88; 95% CI, 0.81–0.96) versus nonusers. A meta-analysis of cohort studies conducted by Ye and Song[100] found a modest risk reduction in CVD associated with higher intakes of vitamins C and E and β-carotene. Results from the prospective Nurses' Health Study showed that long-term use (>15 years) of MVMS (with folic acid) was associated with a decreased risk of colon cancer (RR, 0.25; 95% CI, 0.13–0.51) versus nonuse.[101] A long-term prospective cohort study of subjects enrolled in PHS I found no association between MVMS use and most assessed cardiovascular outcomes, although a 14% decrease was observed in cardiovascular revascularization risk (hazard ratio [HR], 0.86; 95% CI, 0.75–0.98) versus nonuse. In addition, a self-reported history of ≥20 years' MVMS use was associated with a 44% reduction in risk for CVD (HR, 0.56; 95% CI, 0.35–0.90).[102]

Similar to observational studies, RCTs of MVMS use and chronic disease risk have shown mixed results. A meta-analysis of RCTs conducted by Fortman et al[103] indicated that long-term MVMS use (11.2–12.5 years) reduced the incidence of cancer (RR, 0.94; 95% CI, 0.89–1.00) versus placebo. Two studies included in this meta-analysis (PHS II[93] and Supplementation en Vitamines et Mineraux Antioxydants [SU.VI.MAX][104]) individually found a reduction in cancer risk and mortality in men. In PHS II, a significant 8% reduction in total cancer incidence was observed versus placebo (HR, 0.92; 95% CI, 0.86–0.998), as well as an 18% reduction in total cancer incidence in men aged ≥70 years (HR, 0.82; 95% CI, 0.72–0.93).[93] In the total population, the risk for cancer development was 12% lower with MVMS use compared with placebo when prostate cancer was excluded (HR, 0.88; 95% CI, 0.79–0.98). The greatest total cancer risk reduction was observed in the total population of men with baseline cancer histories (HR, 0.73; 95% CI, 0.56–0.96), but no

benefit was observed for specific cancer types (ie, prostate, lung, colorectal, and pancreatic, for which PHS II was insufficiently powered).

Another meta-analysis by Fortman et al[103] reported no significant effect of MVMS for reducing CVD risk (RR, 1.02; 95% CI, 0.94–1.10) based on the results of PHS II and SU.VI.MAX. Other MVMS meta-analyses that evaluated CVD have also primarily reported null outcomes. Six reviews or meta-analyses, principally of RCTs, that evaluated combinations of vitamins C and E, β-carotene, and selenium found no effect on the primary or secondary prevention of CVD.[105–110] Similarly, SU.VI.MAX, which included 13,017 French men and women who received a combination of vitamins C and E, β-carotene, selenium, and zinc, found no significant overall benefit versus placebo in ischemic CVD (RR, 0.97; 95% CI, 0.77–1.20).[111] However, the overall mortality risk was significantly lower in men (RR, 0.63; 95% CI, 0.42–0.93) but not in women (RR, 1.03; 95% CI, 0.64–1.63). In 2 analyses from PHS II, no reduction in major cardiovascular events was observed with higher doses of vitamin C or E for 8 years or MVMS for 11 years,[112,113] but a significant reduction in myocardial infarction death (HR, 0.61; 95% CI, 0.38–0.995) was observed with the MVMS.[113]

PHS II also evaluated subjects on age-related eye disease outcomes and reported a reduction in cataract development (HR, 0.91; 95% CI, 0.83–0.99) and cataract surgeries (HR, 0.89; 95% CI, 0.80–0.99) versus placebo.[114] Similarly, in the Age-Related Eye Disease Study (AREDS), a risk reduction for any lens (OR, 0.88; 95% CI, 0.79–0.98) or nuclear opacity (OR, 0.78; 95% CI, 0.68–0.89) was observed in users of MVMS versus nonusers.[115] The Italian-American Clinical Trial of Nutritional Supplements and Age-Related Cataract administered MVMS for 9 years (N = 1020) and reported a decrease in nuclear opacity progression among a population with early or no cataracts.[116] The AREDS supplement containing vitamins C and E plus zinc, copper, and β-carotene reduced the risk for loss of visual acuity and progression to advanced age-related macular degeneration (ARMD) versus placebo.[117]

Despite the promising data in this area, several observational studies and RCTs have failed to show that MVMS decrease the risk of CMCs. However, some studies conducted with supra-dietary doses of individual micronutrients have indicated the potential for harm (eg, an increased risk of lung cancer with 20 mg β-carotene in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study[118] and an association between high vitamin B_6 single supplements and lung cancer in the Vitamins and Lifestyle Study cohort[119]). Long-term use of MVMS at doses approximating recommended intakes has been shown to be safe.[94] The totality of these data, therefore, is insufficient to support using MVMS for CMC prevention.

8. MVMS use in populations with inadequate intakes or increased needs of micronutrients can provide benefits to apparently healthy individuals, including children, pregnant women, and older adults.

A+: 36%; **A:** 50%; **U:** 14%

Searches for systematic reviews and meta-analyses published in the past 5 years were conducted by using the following criteria: population (adults, children, pregnant women); interventions (MVMS, vitamin D, iron at supplement doses); comparisons (no MVMS); and outcomes (growth, pregnancy outcomes, intelligence/cognition, psychological features, cataracts, safety).

Children

Administration of MVMS and supplements containing vitamin A and zinc can improve linear growth in school-aged children and cognitive performance in children likely to be deficient in micronutrients but otherwise healthy.[120,121] A meta-analysis by Roberts and Stein[120] examined baseline height-for-age *z* score, subject age, nutrient dose, and study duration for heterogeneity in 69 RCTs, most of which were conducted in low- and middle-income countries. Zinc (17 studies and 19 datasets; mean effect size [ES], 0.15; 95% CI, 0.06–0.24), vitamin A (5 studies and 16 datasets; mean ES, 0.05; 95% CI, 0.01–0.09), and ≥2 of any micronutrients (17 studies; mean ES, 0.26; 95% CI, 0.13–0.39) had positive effects on linear growth, but iron, calcium, iodine, and food-based interventions did not. Baseline age, study duration, and dose were not predictors of ES for any nutrient examined. A systematic review of 19 RCTs by Lam and Lawlis[121] conducted with data primarily from developing countries and children experiencing deficiencies found that micronutrients positively affected fluid intelligence (ie, problem solving, logic). No meta-analysis was possible owing to differences in reporting. No consistent effect on

crystallized intelligence (memory), attention, or school performance was found. An examination of MVMS use in infants was not conducted.

Healthy Adults

A meta-analysis of 8 MVMS RCTs conducted by Long and Benton[122] reported a reduction in minor psychiatric symptoms among healthy adults. Perceived stress (standard mean difference [SMD], 0.35; 95% CI, 0.47–0.22; $P = 0.001$), mild psychiatric symptoms (SMD, 0.30; 95% CI, 0.43–0.18; $P = 0.001$), and subclinical anxiety (SMD, 0.32; 95% CI, 0.48–0.16; $P < 0.001$) were reduced compared with placebo, but subclinical depression was not (SMD, 0.20; 95% CI, 0.42–0.03; $P = 0.089$). Fatigue (SMD, 0.27; 95% CI, 0.40–0.146; $P < 0.001$) and confusion (SMD, 0.225; 95% CI, 0.38–0.07; $P < 0.003$) were also reduced compared with placebo.

Pregnant and Breastfeeding Women

Two Cochrane reviews examined MVMS versus iron and/or folic acid supplementation alone during pregnancy. Haider and Bhutta[123] identified 19 RCTs conducted in predominantly low- and middle-income countries (United Kingdom, 2 studies; France, 1 study). A significant reduction in low-birth-weight infants was found with MVMS versus iron and/or folic acid (RR, 0.88; 95% CI, 0.85–0.91), but no differences were found in preterm births or stillbirths, neonatal death, miscarriage, or operative delivery risk. Balogun et al[124] indicated that taking any vitamin supplements before or during early pregnancy did not decrease miscarriage rates.

A meta-analysis of 31 observational studies and 4 RCTs conducted by Wolf et al[125] evaluated the effect of MVMS on pregnancy outcomes in developed countries. Using the GRADE (Grades of Recommendation Assessment, Development, and Evaluation) criteria, the quality of evidence was assessed as low or very low for all outcomes except for NTD recurrence, for which a moderate benefit was found. MVMS use did not change the risk for preterm birth (RR, 0.84; 95% CI, 0.69–1.03). However, the risk of small for gestational age infants (RR, 0.77; 95% CI, 0.63–0.93), NTDs (RR, 0.67; 95% CI, 0.52–0.87), cardiovascular defects (RR, 0.83; 95% CI, 0.70–0.98), urinary tract defects (RR, 0.60; 95% CI, 0.46–0.78), and limb deficiencies (RR, 0.68; 95% CI, 0.52–0.89) decreased.

Vitamin D alone (200–2000 IU/d) or single doses of 60,000 to 600,000 IU were examined in a Cochrane review conducted by De-Regil et al.[126] Data from 477 women (3 RCTs) indicate that vitamin D supplementation during pregnancy reduces the incidence of preterm birth (8.9%) versus no intervention or placebo (15.5%; RR, 0.36; 95% CI, 0.14–0.93; moderate quality). Data from 493 women (3 RCTs; 1 of the aforementioned trials and 2 others) also revealed that vitamin D supplementation during pregnancy reduces the frequency of having a low-birth-weight infant (< 2500 g) compared with no intervention or placebo (RR, 0.40; 95% CI, 0.24–0.67; moderate quality).

Calcium supplementation alone has also been studied at doses typically formulated in MVMS. A Cochrane review by Hofmeyr et al[127] found a small effect of calcium supplementation on pre-eclampsia risk, but these studies were small and the risk for hemolysis, elevated liver enzyme levels, and low platelets increased with supplementation.

Older Adults

A Cochrane review that examined vitamin D supplementation versus placebo or no intervention found a decrease in mortality when all 56 trials were analyzed together (5920 of 47,472 [12.5%] vs 6077 of 47,814 [12.7%]; RR, 0.97; 95% CI, 0.94–0.99; $P = 0.02$; $I^2 = 0\%$).[128] However, these studies were of low to moderate quality due to considerable attrition and the relatively short duration of some studies, and a sensitivity analysis suggested that the result should be considered with caution. A meta-analysis of 24 RCTs by Forbes et al[129] involving interventions including B vitamins, vitamin E, or omega-3 fatty acids evaluated cognitive function and found no statistically significant effect on Mini–Mental State Examination or digit span forward scores. There is evidence, primarily from cohort studies, that MVMS may reduce cataract risk.[130]

9. Some individuals with CMCs experience nutritional deficiencies and/or inadequacies that can be prevented and treated with adequate dietary management and/or the use of MVMS.

A+: 86%; **A:** 14%

The evidence reviewed here included studies identified through a PubMed search; diverse ranges of search terms related to MVMS and some specific CMCs were included.

Age-related Eye Diseases

In the AREDS 2 study, 1 supplement replaced β-carotene with lutein and zeaxanthin, and a significant benefit ($P = 0.01$) was observed in slowing the progression to advanced ARMD in patients with low dietary intake of these carotenoids and either current bilateral large drusen or large drusen in 1 eye and advanced ARMD in the other eye.[131] In the Lutein Antioxidant Supplementation Trial, subjects with existing atrophic ARMD who received lutein alone or combined with antioxidants showed improvements in visual function after 1 year.[132]

Women's Health

Female infertility treatment has been shown to improve with micronutrient supplementation, particularly with combinations of folic acid; vitamins B_6, C, and D; iodine; selenium; iron; and/or omega-3 fatty acids.[133] In a small pilot study, MVMS have shown better results versus folic acid alone in ovulation induction among women undergoing fertility treatment.[134] Osteopenia and osteoporosis are prevalent in postmenopausal women, and optimization of vitamin D and calcium intake is recommended for managing individuals with these conditions due to their role in maintaining bone mineral density and reducing the risk of falling[135,136]; however, those at highest risk of subsequent fractures will likely require additional pharmacologic and nonpharmacologic treatments.

Obesity

Deficiencies or inadequacies in vitamin B_6, C, D, and E can occur among obese individuals,[137] and many weight-loss diets can cause micronutrient inadequacies.[138] Furthermore, body mass index has been shown to be associated with poor folate status in nonpregnant women of childbearing age, suggesting that obesity may modify folate metabolism.[139] Bariatric surgery can also cause or exacerbate micronutrient deficiencies, especially vitamin B_{12} and iron, and this risk is higher with gastric bypass procedures that involve food malabsorption.[140] After bariatric surgery (particularly Roux-en-Y gastric bypass), dietary patterns do not improve to an ideal level.[141] Vitamin D deficiency after bariatric surgery is common and must be treated to reduce osteoporosis risk.[142] Preventing micronutrient deficiencies is believed to be critical to bariatric surgery success.[143] This scenario may be particularly important for obese women of childbearing potential who may be at an increased risk for key nutrient deficiencies and inadequacies related to negative pregnancy outcomes (eg, NTDs).[144] Although most patients receive post–bariatric-surgery MVMS and report improvements in their nutritional status and prevention of anemia, there is a large disparity in the prevalence of this practice.[145,146] Furthermore, MVMS may not provide adequate nutritional support,[147] and individual supplements at higher doses may be necessary for certain individuals.

Type 2 Diabetes Mellitus

Levels of vitamins A, C, and E, thiamine, pyridoxine, and biotin can be reduced in type 2 diabetes mellitus (T2DM), and metformin treatment impairs the bioavailability of vitamin B_{12} and folic acid.[148,149] Metformin also negatively affects vitamin B_{12} status in patients with polycystic ovary syndrome.[150] Most MVMS studies in patients with diabetes have shown inconclusive results on disease progression and its sequelae, although MVMS improved micronutrient status.[148] However, a meta-analysis of RCTs that evaluated magnesium supplementation reported improvements in insulin resistance.[151] An RCT conducted in patients with T2DM who received MVMS with a zinc supplement reported improvements in glycemic control and lipid profile versus placebo.[152]

DISCUSSION

Health care research and policy development are primarily driven by the paradigm of evidence-based medicine. However, certain facets of nutrition research regarding health promotion and disease prevention, as implemented, do not fit well within this context; in particular, reliance on RCTs as the "gold standard" for evidence-based policy.[153–156] For example, although nutritional status at baseline and dietary variability during the RCTs (especially when these factors are not assessed) can significantly affect the interpretation of results, these are often overlooked in the analysis. In addition, dietary supplements, MVMS in particular, typically provide doses that approximate recommended intake levels and would only produce modest changes, albeit with potentially large public health benefits. Inappropriately conflating expectations regarding health outcomes of nutritional interventions can lead to confusion among HCPs

and incorrect conclusions, and findings arising from the research can often be misrepresented in the lay media, resulting in misunderstandings by the public.

The conflicting evidence associated with the use and benefits of MVMS helps reveal several gaps in our knowledge base and provides a rationale for convening this consensus panel. As suggested in these summaries, although the essentiality of micronutrients in human biology and health is well understood, additional research is necessary to fully elucidate the role of MVMS for maintaining and promoting health and preventing CMCs. Despite certain limitations associated with various research approaches in several of the studies described here, there is a clear indication that, within the general population, appropriately formulated MVMS can safely provide essential micronutrients to help individuals achieve recommended intake levels.[28,94] However, when recommending any dietary supplement to a patient, it is important that HCPs consider individual factors, including dietary micronutrient intakes, to avoid exceeding ULs of nutrients that may cause adverse effects when overconsumed. As noted earlier, MVMS should not be viewed as substitutes for a balanced diet but should be recommended, in addition to other advice for a healthy lifestyle, to ensure adequate micronutrient intake and status.

CONCLUSIONS

This consensus panel has indicated that MVMS can improve the micronutrient intake and, hence, the nutritional status of individuals presenting with deficiencies and inadequacies, including those with CMCs. However, the effect of MVMS on the primary prevention of CMCs is presently inconclusive, despite some modest yet promising results from RCTs. Importantly, there is a clear indication that the long-term use of MVMS formulated with doses that do not exceed ULs is safe; however, additional research is necessary to fully define the benefits of MVMS for health promotion and disease prevention. Consumers and clinicians should therefore consider the risks of deficiencies and the potential benefits of supplementation. Given the relatively low cost and established safety of MVMS, as well as the essentiality of adequate micronutrient status for human biology and good health, HCPs should assess their patients' dietary needs and risk of micronutrient inadequacies and consider intervening with MVMS for their at-risk patients.

CONFLICTS OF INTEREST

At the direction of the co-chairs (Drs. Blumberg and Cena), after an initial discussion with Pfizer Consumer Healthcare regarding the project, the company played no role in the Delphi process or preparation of this report but did provide funding for the organizational aspects of the Delphi process, the panel meeting in Philadelphia, and the contributions of Peloton Advantage, which provided overall coordination of the process and editorial assistance.

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Dr. Blumberg acts as a consultant to companies that manufacture or market dietary supplements, including service on the Nutrition Advisory Committee at Pfizer Consumer Healthcare. Dr. Cena acts as a consultant to companies that manufacture or market dietary supplements, including Pfizer Consumer Healthcare. Dr. Biesalski acts as a consultant for Ratiopharm, a company that manufactures dietary supplements. Dr. Frei acts as a consultant for Pfizer Consumer Healthcare and DSM Nutritional Products. Dr. Hwalla acts as a consultant for Pfizer Consumer Healthcare. Dr. Lategan-Potgieter has received honoraria from Pfizer Inc. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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Drs. Blumberg and Cena co-chaired the consensus panel meetings; they directed and participated in the development of the statements, the consensus process, drafting of evidence reviews, and preparation of the manuscript. All the authors contributed to the development of the manuscript through reviews, revisions, and approval of the final version.

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Review

Meta-Analysis of the Effects of Foods and Derived Products Containing Ellagitannins and Anthocyanins on Cardiometabolic Biomarkers: Analysis of Factors Influencing Variability of the Individual Responses

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Abstract: Understanding interindividual variability in response to dietary polyphenols remains essential to elucidate their effects on cardiometabolic disease development. A meta-analysis of 128 randomized clinical trials was conducted to investigate the effects of berries and red grapes/wine as sources of anthocyanins and of nuts and pomegranate as sources of ellagitannins on a range of cardiometabolic risk biomarkers. The potential influence of various demographic and lifestyle

factors on the variability in the response to these products were explored. Both anthocyanin- and ellagitannin-containing products reduced total-cholesterol with nuts and berries yielding more significant effects than pomegranate and grapes. Blood pressure was significantly reduced by the two main sources of anthocyanins, berries and red grapes/wine, whereas waist circumference, LDL-cholesterol, triglycerides, and glucose were most significantly lowered by the ellagitannin-products, particularly nuts. Additionally, we found an indication of a small increase in HDL-cholesterol most significant with nuts and, in flow-mediated dilation by nuts and berries. Most of these effects were detected in obese/overweight people but we found limited or non-evidence in normoweight individuals or of the influence of sex or smoking status. The effects of other factors, i.e., habitual diet, health status or country where the study was conducted, were inconsistent and require further investigation.

Keywords: ellagitannins; anthocyanins; interindividual variability; meta-analysis; cardiometabolic disorders; pomegranate; nuts; berries; red wine; red grapes

1. Introduction

Cardiometabolic dysfunction is diagnosed in about 8% of the global adult population and is characterized by dyslipidemia, hypertension, obesity, glucose intolerance and insulin resistance [1]. It is well recognized that a diet rich in plant-based foods helps prevent or reduce these cardiometabolic disorders and that increasing the intake of fruits, vegetables, cereals and nuts constitutes part of the strategy to combat these disorders [2,3]. Plant foods provide a variety of micro- and macronutrients, i.e., minerals, vitamins, fibers, proteins as well as a range of bioactive compounds that are beneficial for our health [4]. Over the past few decades, a major area of research has specifically focused on the study of the plant-derived bioactive compounds, such as polyphenols, and their cardiometabolic protective properties in humans which is summarized by Lecour et al. [5].

A variety of fruits and nuts are good sources of different polyphenols including anthocyanins (ANCs) and ellagitannins (ETs). In particular, berries, red grapes and red wine are important sources of ANCs [6,7]. The main ANCs present in our diet are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin [8,9]. Cyanidin-3-glucoside is the most frequent ANC found in raspberries, blackberries, elderberries, purple corn or black carrots. Moreover, malvidin-3-glucoside is the major ANC in red grapes and wines whilst pelargonidin-3-glucoside in strawberries. Blueberries, very often used in intervention studies, contain a mixture of delphinidin, malvidin and cyanidin derivatives [8,10]. The highest ANCs content is found in chokeberry (400 to 1500 mg/100 g fresh weight (F.W.)), blackcurrant (100 to 500 mg/100 g F.W.), blackberries (50 to 350 mg/100 g F.W.), blueberries (60 to 300 mg/100 g F.W.) and purple corn ($\geq$1500 mg/100 g F.W.) [8,11]. On the other hand, pomegranate and nuts contain important quantities of ETs (150–500 mg/100 mL pomegranate juice; ~1600 mg/100 g walnuts) although they can also be found in berries at lower concentrations (50–350 mg/100 g F.W. raspberry; 25–80 mg/100 g F.W. strawberry) [12–14]. The ETs most commonly ingested by humans are punicalagin, peduncalagin and sanguiin [15].

Previous reviews and meta-analyses of randomized-controlled trials (RCTs) have explored the evidence of the effects of the intake of berry [16–21], nut [22,23], pomegranate [24,25], and grape [26,27] foods and (or) derived products on different cardiometabolic risk factors (i.e., serum lipids, blood pressure, glucose). The results of these analyses have indicated inconsistencies in the overall effects and have pointed at potential different responses between different subpopulations. Some of the reasons for the lack of consistent results might be the insufficient number of the RCTs included in these meta-analyses as well as their heterogeneity and inadequate description of the study population. There are important differences between studies for a number of key factors (body mass index (BMI), sex,

smoking habits, diet, health status) that likely influence the response of the participants to the intake of the compounds tested. These differences can mask significant effects in specific populations [28,29].

Another critical issue is the test products used in those RCTs. In general, these studies have been conducted with different types of foods or derived processed products (extracts, drinks, freeze-dried products) with different origin, quality and composed by mixtures of diverse components often not fully characterized. It is thus difficult to attribute the beneficial effects to a particular compound(s). In this regard, some studies have been conducted with polyphenol-rich berry products in comparison with nutrient-matched controls to try to associate the polyphenols intake with the response of the individuals [30]. Also, the association between the intake of ET-containing pomegranate products and the effects on cardiometabolic risk factors has been investigated with some evidence of a potential link between responses and specific ET-derived metabolites, but the effects of other constituents of these products have not been fully discarded [31,32]. At present, specific associations between pure ANCs or ETS and cardiometabolic effects remain unproved.

As the number of RCTs published increases, it is thus important that: (i) we continue evaluating the accumulated overall evidence that support the benefits of the plant bioactive compounds on cardiometabolic health and (ii) we try to elucidate the contribution of the factors that determine interindividual variability in response to the intake of these plant-derived bioactive compounds. Eventually, we shall be able to understand and establish the true effectiveness of these compounds against cardiometabolic disease [33]. Along these lines, the main goals of the present study were: (i) to systematically review and appraise through meta-analysis, all available human RCTs that have investigated the association between the intake of various foods (berries, red grapes and wine, pomegranate and nuts) as well as of their derived products (extracts, powders, drinks), as sources of ANCs and ETs, on 13 biomarkers of cardiometabolic risk; and (ii) to provide an in-depth evaluation of the potential influence of a range of key factors on the interindividual variability in response to the intake of these products.

2. Results

2.1. Description of the Selected Studies

A total of 2374 articles were initially selected through the search on the electronic databases (Medline (PubMed) and Web of Science). After removal of duplicates, screening and application of exclusion criteria, 241 trials were selected for data extraction. After detailed analysis of the full text, 113 articles were rejected, due to lack of relevant outcomes, aspects of the study design, etc. Finally, 128 human RCTs published between March 1995 and March 2016 (included) [34–160] were incorporated in this systematic review and meta-analysis. A flow diagram with the details of the study selection is shown in Figure 1.

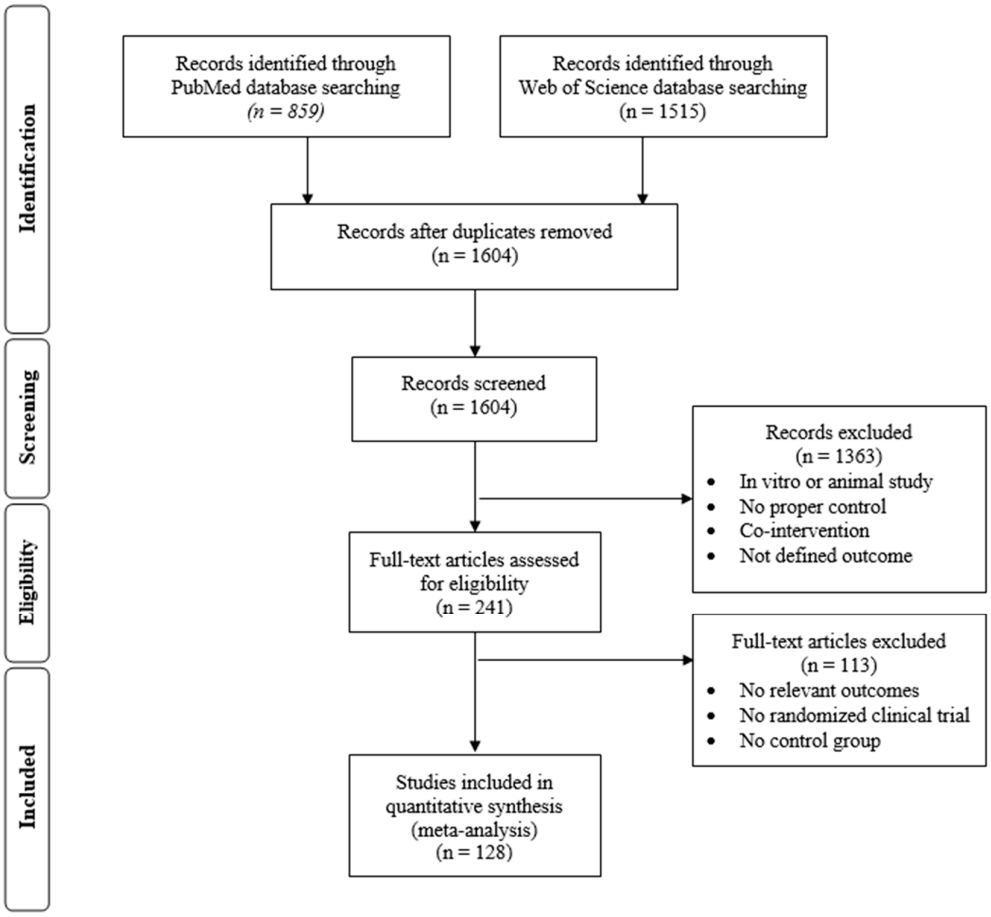

Figure 1. Flow diagram showing the study selection process.

2.2. Characteristics and Quality of the Included Studies

The 128 RCTs included a total of 5538 participants from countries distributed over five continents as follows: 1500 participants from Asia, 1731 from North-America, 119 participants from South America (Chile), 1830 participants from Europe, 64 participants from South Africa, and 294 participants from Oceania (Australia and New Zealand). Of all the studies, 30 RCTs (1542 total participants) were conducted with foods and derived products containing ETs as the main polyphenols, i.e., pomegranate and nuts (walnuts, almond, pistachios, peanuts, pine nuts, hazelnuts) and 99 RCTs (4086 total participants) were conducted with foods and food products considered rich sources of ANCs (berries, red grapes, red wine). The test products were provided as a liquid (drinks, beverages, juices) or a solid (powder or extracts in capsules, tablets, foods). Intervention doses ranged between 30 g and 230 g for nuts, between 100 mL and 500 mL for pomegranate juice and between 435 mg and 700 mg for pomegranate extracts. RCTs conducted with berries had doses ranging from 80 mg to 38 g for extracts and from 230 mL to 750 mL for beverages or juices. Regarding red wines or grapes doses ranged from 100 mg to 2 g for extracts and from 250 to 400 mL for drinks. The participants in these RCTs represented a mixed population of men and women ranging from young adults to elderly participants, and with a higher prevalence (~60%) of individuals with a BMI $\geq$ 25.0 kg/m^2 (overweight and (or) obese volunteers). Only 28 and 9 RCTs were conducted separately with men and women, respectively. Most studies failed to report the smoking habits of participants; when reported, participants were typically non-smokers (~35%) or a mixed sample population (~50%). With regards to the health status, the sample population constituted of healthy individuals (1764 participants), overweight and (or) obese individuals but not medicated (classified as 'at risk'; 870 participants), as well as individuals

with an incipient or with a reported chronic risk factor or metabolic disease (2804 participants). Among these, some participants were taking medication, others were not medicated or medication use was not reported. Most interventions (~80%) ranged from 1 week to 3 months during which the participants followed either a controlled diet (~40%) or their habitual diets (~60%). RCTs conducted for more than 3 months or acute studies represented each ~10% of the total number of interventions. Most of the studies (~62%) were classified as studies with a moderate to low risk of bias (score $\geq$ 5.0 and <8.0 or $\geq$8.0 and $\leq$10.0, respectively), while ~38% of the studies obtained a low score (<5.0) and were considered as a high risk of bias. A list of all the studies included in this meta-analysis, their characteristics and corresponding risk bias score is included in Supplementary Table S1.

2.3. Overall Impact of Supplementation with Foods and Derived Products Containing ETs and/or ANCs on Biomarkers of Cardiovascular Risk

Initial analysis examined the effects of the supplementation with ET- and/or ANC-containing foods and/or derived products on the list of selected biomarkers of cardiometabolic risk at a total population level. A substantial number of RCTs (18 to 109 depending on the variable investigated) including a total number of participants (n = 563 to 3991) with highly variable heterogeneity ($I^2 \approx$ 25.0 to 93.0%) were included in the analyses. Forest plots and Funnel plots representing all the individual studies and the overall impact of the supplementation with these products on each biomarker are shown in Supplementary Figures S1–S39. Visual inspection of the Funnel plots showed symmetrical shapes and absence of publication bias in most of the variables investigated, however, we detected some asymmetry in the case of LDL-C, HDL-C, TAGs, and FMD (SDM values) and of DBP (DM values). All these results were further confirmed by Egger's regression. Supplementary Tables S2 and S3 display the overall results as standardized difference in means (SDM) and difference in means (DM), respectively, using the random model. A summary listing the significant effects expressed as SDM and DM values as well as their corresponding 95% confidence intervals and GRADE quality of evidence is presented in Table 1.

Among the 13 cardiometabolic outcomes investigated, we observed a significant evidence of the reduction in WC, T-C, SBP and DBP following supplementation with the ET- and/or ANC-containing products. Additionally, we detected an increase in HDL-C further supported by a significant although small positive relationship with the duration (days) of the supplementation (SDM random-effects meta-regression; slope: 0.002; p-value: 0.004) (Supplementary Figure S40). FMD was also consistently increased by the treatment with these compounds although it was only statistically significant when the effects were calculated as DM. The quality of the evidence was evaluated as high for blood pressure, moderate for WC, T-C, HDL-C and low for FMD due to many studies reporting serious risk of bias across studies. We additionally detected a small reduction, although not significant, in glucose (SDM: $-$0.101, p-value = 0.095) and TAGs (DM: $-$0.006, p-value = 0.086) whereas BMI, LDL-C, insulin, HbA1c, and HOMA-IR were seemingly not affected by the intervention with these types of products.

We next compared the effects on all the biomarkers between those foods and/or products that are richer sources of ETs, nuts and pomegranate (subgroup referred as to ETs) and those foods and products that contain higher levels of ANCs, mostly berries, red grapes, red wine (subgroup referred as to ANCs). Supplementary Table S4 includes the random effects (SDM and DM) for each separate group and the comparison between them (ETs vs. ANCs sources) for all the investigated biomarkers. A summary with the most significant effects is presented in Table 2.

Table 1. Summary of the most significant global effects of foods and food products containing ETs and ANCs on biomarkers of cardiometabolic risk.

	SDM (*p*-Value)	95% CI	*n*	N_T	I^2 (%)	GRADE [1]		DM (*p*-Value)	95% CI	*n*	N_T	I^2 (%)	GRADE
WC	−0.30 (0.008)	(−0.52, −0.08)	23	1023	63.4	Moderate [2]	WC (cm)	−1.22 (0.005)	(−2.07, −0.36)	22	972	37.5	Moderate [2]
T-C	−0.17 (0.001)	(−0.27, −0.07)	109	3991	54.5	Moderate [2]	T-C (mmol/L)	−0.10 (0.013)	(−0.18, −0.02)	103	3673	70.6	Moderate [2]
SBP	−0.20 (0.000)	(−0.28, −0.12)	95	3539	25.0	High	SBP (mm Hg)	−1.56 (0.000)	(−2.13, −0.99)	83	3175	0.00	High
DBP	−0.19 (0.000)	(−0.26, −0.11)	99	3790	27.9	High	DBP (mm Hg)	−1.42 (0.000)	(−2.08, −0.76)	90	3473	41.6	Moderate [2]
HDL-C	+0.11 (0.034)	(0.01, 0.21)	99	3581	53.1	Low [2,3]	HDL-C (mmol/L)	+0.03 (0.062)	(0.00, 0.05)	92	3239	61.6	Moderate [2]
FMD	+0.20 (NS)	(−0.17, 0.57)	22	563	73.8	Low [2,3]	FMD (%)	+0.64 (0.027)	(0.07, 1.20)	21	547	82.2	Low [2,3]

Significant: *p*-value 0.05; Marginally Significant (0.05 ≤ *p*-value 0.1). SDM: standardized difference in means; DM: Difference in means; CI: confidence intervals; *n*: total number of studies; N_T: total number of participants; I^2: Heterogeneity Index; WC: Waist Circumference; T-C: Total Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; FMD: Flow Mediated Dilation. [1] GRADE quality of evidence downgraded from high to moderate or low in the presence of serious risk of bias across studies and (or) serious risk of reporting bias; [2] serious risk of bias across studies: more than 50% of the studies had unclear allocation concealment, no double-blind studies and unclear reporting of dropouts; [3] serious risk of reporting bias: Eggert *p*-value 0.05 and more than 50% of small studies with limited number of participants.

Table 2. Summary of the most significant effects of the foods and food products containing ETs (pomegranate and nuts) and those containing ANCs (berries, red grapes and red wine) on biomarkers of cardiometabolic risk.

ET-Containing Products (Pomegranate, Nuts)						ANC-Containing Products (Berries, Red Grapes, Red Wine)					
	SDM (*p*-Value)	*n*		DM (*p*-Value)	*n*		SDM (*p*-Value)	*n*		DM (*p*-Value)	*n*
WC	−0.70 (0.025)	7	WC (cm)	−1.53 (0.031)	6	WC	−0.12 (NS)	16	WC (cm)	−0.75 (NS)	16
T-C	−0.18 (0.006)	28	T-C (mmol/L)	−0.09 (0.000)	26	T-C	−0.17 (0.008)	81	T-C (mmol/L)	−0.10 (0.094)	77
HDL-C	+0.10 (NS)	23	HDL-C (mmol/L)	+0.03 (NS)	21	HDL-C	+0.12 (NS)	76	HDL-C (mmol/L)	+0.03 (NS)	71
LDL-C	−0.19 (0.031)	26	LDL-C (mmol/L)	−0.11 (0.000)	24	LDL-C	0.05 (NS)	71	LDL-C (mmol/L)	−0.03 (NS)	68
TAGs	−0.24 (0.025)	26	TAGS (mmol/L)	−0.11 (0.000)	24	TAGs	+0.004 (NS)	71	TAGS (mmol/L)	+0.02 (NS)	64
SBP	−0.11 (NS)	21	SBP (mm Hg)	−1.89 (NS)	15	SBP	−0.23 (0.000)	74	SBP (mm Hg)	−2.19 (0.000)	68
DBP	−0.14 (NS)	20	DBP (mm Hg)	−1.28 (NS)	18	DBP	−0.20 (0.000)	79	DBP (mm Hg)	−1.58 (0.000)	72
FMD	+0.62 (0.014)	3	FMD (%)	+0.39 (NS)	3	FMD	+0.12 (NS)	19	FMD (%)	+0.53 (NS)	18
Glucose	−0.24 (0.052)	16	Glucose (mmol/L)	−0.12 (0.01)	15	Glucose	−0.05 (NS)	22	Glucose (mmol/L)	+0.001 (NS)	45

Significant: *p*-value 0.05; Marginally Significant (0.05 ≤ *p*-value 0.1). WC: Waist Circumference; T-C: Total Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; TAGs: Triglycerides; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; FMD: Flow Mediated Dilation; *n*: total number of studies included in the analysis; SDM: standardized difference in means; DM: Difference in means.

Overall, there was a higher number of clinical trials looking at the cardiometabolic regulatory effects of ANC-rich berries, grapes and wine (n = 99) than studies carried out with pomegranate or nuts (n = 30). Regardless of the number of studies per subgroup, stratification into these two types of products did not alter the significant effect on T-C levels. However, the analysis of the separate subgroups highlighted some dissimilarity between the supplementation with the two types of products, with the ET-containing pomegranate and nuts being more effective (i.e., greater effect size and more significant results) than the ANCs subgroup at reducing WC (subgroups comparison (SDM): Q statistic = 6.70, p-value = 0.01), LDL-C, TAGs (subgroups comparison (SDM): Q statistic = 4.06, p-value = 0.044) and glucose. On the other hand, the ANC-rich products were significantly effective at lowering both SBP and DBP, whereas this was not the case in the ETs subgroup. We cannot ignore, however, that this may be due to the smaller number of studies carried out with the ETs-containing products. Also, probably due to the smaller number of studies per subgroup, the initially observed significant increase in HDL-C (all products together) was not significant in each separate subgroup. In addition, we found a significant increase in FMD (SDM) in the ETs subgroup, though these results should be interpreted cautiously since the number of trials was very small (n = 3). Given some of the differences found between the ETs and ANCs subgroups, we next carried out the rest of the stratification analyses in each separate subgroup.

2.4. Comparative Analysis of the Potential Factors Influencing Interindividual Variability in the Responses to the Consumption of Foods and Food Products Containing ETs and ANCs

2.4.1. Stratification by the Individuals' Baseline BMI, Sex, Smoking Habits and Background Diet

Stratification by baseline BMI values: <25.0 Kg/m^2 (normal individuals) vs. ≥25.0 Kg/m^2 (overweight and obese individuals) (Supplementary Table S5) evidenced a general absence or a small number of studies carried out in normal individuals in the ETs subgroup (≤3) and in the ANCs subgroup (between 5 and 13) for most of the biomarkers investigated. Neither ETs nor ANCs had any effect on the biomarkers investigated in the normoweight subpopulation. The most noteworthy effects of the ETs- and ANCs-containing products in overweight and obese individuals are summarized in Table 3.

The reduction of T-C by these two types of products remained significant in the individuals with a baseline BMI ≥ 25.0 Kg/m^2 although the extent of the reduction appeared to be smaller in the ETs subgroup. The reduction of WC, LDL-C, TAGs and glucose by the ETs-containing pomegranate and nuts in the total population (Table 2) was still seen in the overweight/obese subgroup. For FMD, we found that the 3 studies included in the analysis of the ETs subgroup were all carried out in overweight/obese individuals and thus the results are the same as in Table 2. Regarding blood pressure, the ANC-containing products were also effective at lowering SBP and DBP in the overweight/obese people. Of note, the ET-products which did not significantly affect blood pressure in the overall population group (Table 2) became effective and significant at lowering the SBP in the overweight/obese people.

Regarding sex stratification, there were also few studies specifically carried out with either only men (n = 1–24) or women (n = 1–9) depending on the product and biomarker and thus, the evidence for the effect of sex in the response to ETs or ANCs was limited and most results were not significant (Supplementary Table S6). We found, however, that the reduction of DBP in men by ANCs was still significant (SDM: −0.19, p-value = 0.017, n = 24; DM: −1.70 mm Hg, p-value = 0.012, n = 22) whereas in women the results did not reach significance (SDM: −0.19, p-value = 0.092, n = 8; DM: −1.81 mm Hg, p-value = 0.087, n = 8), possibly due to the small number of studies in the women subgroup.

Table 3. Summary of the most significant effects of the foods and food products containing ETs (pomegranate and nuts) and those containing ANCs (berries, red grapes and red wine) on biomarkers of cardiometabolic risk in overweight and (or) obese individuals (baseline BMI: $\geq$ 25.0 Kg/m^2).

BMI $\geq$ 25.0 kg/m^2	**ET-Containing Products (Pomegranate, Nuts)**					**ANC-Containing Products (Berries, Red Grapes, Red Wine)**				
	SDM (*p*-Value)	***n***		**DM (*p*-Value)**	***n***		**SDM (*p*-Value)**	***n***		**DM (*p*-Value)**	***n***
WC	−0.70 (NS)	4	WC (cm)	−1.71 (0.047)	3	WC	−0.11 (NS)	10	WC (cm)	−0.70 (NS)	10
T-C	−0.12 (NS)	20	T-C (mmol/L)	−0.08 (0.000)	18	T-C	−0.34 (0.003)	35	T-C (mmol/L)	−0.23 (0.009)	35
LDL-C	−0.15 (NS)	20	LDL-C (mmol/L)	−0.11, (0.000)	18	LDL-C	−0.13 (NS)	30	LDL-C (mmol/L)	−0.11 (NS)	29
TAGs	−0.21 (NS)	19	TAGs (mmol/L)	−0.11 (0.000)	17	TAGs	−0.05 (NS)	28	TAGs (mmol/L)	−0.05 (NS)	27
SBP	−0.26 (0.012)	14	SBP (mm Hg)	−3.10 (0.033)	11	SBP	−0.25 (0.000)	43	SBP (mm Hg)	−1.54 (0.000)	38
DBP	−0.08 (NS)	15	DBP (mm Hg)	−0.55 (NS)	14	DBP	−0.22 (0.002)	30	DBP (mm Hg)	−1.62 (0.000)	29
FMD	+0.62 (0.014)	3	FMD (%)	+0.39 (NS)	3	FMD	−0.20 (NS)	6	FMD (%)	−0.65 (NS)	6
Glucose	−0.19 (0.058)	14	Glucose (mmol/L)	−0.18 (0.017)	13	Glucose	−0.13 (NS)	22	Glucose (mmol/L)	−0.02 (NS)	20

Significant: *p*-value 0.05; Marginally Significant (0.05 $\leq$ *p*-value 0.1). WC: Waist Circumference; T-C: Total Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; TAGs: Triglycerides; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; FMD: Flow Mediated Dilation; *n*: total number of studies included in the analysis; SDM: standardized difference in means; DM: Difference in means.

We did not find any study carried out specifically with smokers in the ETs subgroup and only very few studies (n = 1–3) in the ANCs subgroup (Supplementary Table S7). Most studies were conducted with non-smokers or in a mix population. The ET-containing products reduced WC (DM = −1.72, p-value = 0.047, n = 3), DBP (SDM: −0.27, p-value = 0.035, n = 9), and glucose (DM = −0.11, p-value = 0.000, n = 5) in the non-smokers subgroup. In the ANCs subgroup, the reduction of both SBP and DBP remained significant for non-smoker individuals. FMD was significantly reduced by the ANC-containing products in smokers (SDM = −1.61, p-value = 0.000; DM = −3.53%, p-value = 0.000). Statistical comparison between smokers vs. non-smokers confirmed the difference in the effect between the two subgroups ((SDM): Q statistic = 5.93, p-value = 0.015; (DM): Q statistic = 4.84, p-value = 0.028). These results should be interpreted with caution due to the small number of studies included.

Studies were also stratified according to the type of background diet (controlled vs. usual) followed during the supplementation period with the ET- or the ANC-containing products (Supplementary Table S8). In most cases, the results of the analyses were not significant with independence of the type of diet followed during the intervention. The reduction of T-C by the products containing ETs were significant both in trials carried out with the usual diet, as well as those carried out with controlled diet whereas the levels of TAGs and the WC were most significantly reduced only in the subgroup that followed a controlled diet (subgroups comparison (SDM): Q statistic = −4.66, p-value = 0.031 for TAGs; (SDM): Q statistic = 19.52, p-value < 0.001 and (DM): Q statistic = 20.32, p-value < 0.001 for WC). Additionally, we found some indication of the increase of FMD in studies carried out with the usual diet (SDM: +0.62, p-value = 0.014, n = 3) and of the decrease of LDL-C (DM: −0.11 mmol/L, p-value = 0.000, n = 9) and glucose (DM: −0.15, p-value = 0.038, n = 8) under a controlled diet.

Regarding supplementation with ANCs, the reduction of SBP and DBP by these compounds was significant both in interventions maintaining the usual diet and in those carried out with controlled diets. We additionally detected a significant decrease in the LDL-C levels but only in the subgroup that followed the usual diet (SDM: −0.31, p-value = 0.027; DM: −0.22 mmol/L, p-value = 0.016, n = 16–17).

2.4.2. Stratification by the Health Status of the Participants

As for the previous factors examined, stratification of the studies by reported health status of the participants reduced considerably the number of studies in many of the resulting subgroups, as well as the significance of the results (Supplementary Table S9a,b).

In the subgroup containing studies with healthy participants, the evidence of the effects of the ET-containing pomegranate and nuts was limited to a significant reduction of T-C (SDM: −0.21, p-value = 0.021; DM: −0.15 mmol/L, p-value = 0.028, n = 10) and of glucose (SDM: −0.62, p-value = 0.023; DM: −0.23 mmol/L, p-value = 0.016, n = 5) (Supplementary Table S9a). In those studies carried out with volunteers 'at risk', the ET-containing products reduced LDL-C (SDM: −0.55, p-value = 0.030; DM: −0.11 mmol/L, p-value = 0.000, n = 4) and SBP (SDM: −0.40, p-value = 0.004; DM: −1.15 mm Hg, p-value = 0.003, n = 4). There was also a small reduction in T-C (DM: −0.08 mmol/L, p-value = 0.000, n = 4) and in TAGs (DM: −0.11 mmol/L, p-value = 0.000, n = 4) and an increase in HDL-C (SDM: +0.35, p-value = 0.030, n = 6). In the reported disease subgroup, we only detected a small reduction in WC (SDM: −0.57, p-value = 0.065; DM: −0.71 cm, p-value = 0.029) and an increase in FMD (SDM: +0.66, p-value = 0.037). Subgroups comparison also highlighted that the ET-containing products were more effective at reducing glucose in healthy volunteers than in participants with some disease ((SDM): Q statistic = 5.99, p-value = 0.014 and (DM): Q statistic = 3.91, p-value = 0.048). All these results were based on a very small number of studies and should be taken with caution.

Regarding the ANC-containing products, the most clear and consistent evidence was that supplementation with these products significantly reduced blood pressure independent of the health status of the participants and thus, they similarly lowered SBP and DBP in healthy non-medicated individuals, in participants 'at risk' (also non medicated), as well as in those patients with some diagnosed cardiovascular disease and under medication (Supplementary Table S9b). Also, the ANCs

significanlty reduced WC only in the subgroup of 'at risk' participants (SDM: −0.24, p-value = 0.017; DM: −1.72 cm, p-value = 0.064, n = 7) whereas T-C was downregulated in the healthy subgroup (DM: −0.15 mmol/L, p-value = 0.003, n = 32) and in the disease subgroup (SDM: −0.24, p-value = 0.042, n = 36).

We did not find significant evidence in any particular subgroup (healthy, 'at risk', disease) in which the supplementation with ANC-containing products caused a change in BMI, LDL-C, HDL-C, TAGs, glucose, insulin, and HOMA-IR. Instead, we detected a significant increase in FMD in the healthy subgroup (DM: +0.92%, p-value = 0.049, n = 10).

2.4.3. Stratification by the Country Where the Study Was Carried Out

In the absence of proper characterization of the ethnicity of the participants (most studies did not report this information), we explored the potential influence of the country of recruitment or country where the study was carried out. Once more and despite the reduction of the number of studies per subgroup and the limited significance of most results, we were able to find some significant data with sufficient number of studies for some of the outcomes examined (Supplementary Table S10a–c).

There were very few studies (n = 1–4) carried out with ET-containing pomegranate or nuts in East Asian countries. In this subgroup, we only found a significant reduction of T-C (DM: −0.19 mmol/L, p-value = 0.050, n = 4). In the subgroup constituted by all-the-other-countries except the East Asian ones, the reduction of T-C was also significant, as well as that of WC, LDL-C, TAGs and glucose (Supplementary Table S10a). The subgroup of studies carried out in North America (Supplementary Table S10b) also gave some significant evidence of the reduction of WC, T-C, LDL-C, TAGs, and of insulin levels even though the number of studies included was not very big (n = 4–11). Subgroup comparions between studies conducted in North America and those carried out in Europe detected a significant difference in the effects of these products on insuline which was increased in the European studies ((DM): Q statistic = 5.79, p-value = 0.016). In addition, a small but significant increase in FMD was seen in the North American countries but only with two studies included. Overall, the studies conducted in European countries with ET-containing products show limited evidence of the reduction of T-C, LDL-C, glucose and insulin. Of note, the DBP was reduced by this type of product in European countries (DM: −5.21 mm Hg, p-value = 0.048, n = 5). Further stratification into Mediterraean or non-Mediterranean European countries (Supplementary Table S10c) reduced the number of studies per subgroup to 1 or 2 for most of the biomarkers investigated and the results were mostly not significant. Nevertheless, in the Non-Mediterranean countries there was a tendency towards the reduction of T-C, LDL-C, DBP, and glucose by the intake of ET-products. Insulin was significantly augmented in this subgroup (DM: +3.04 mIU/mL, p-value = 0.027) in the limited studies reported (n = 2).

In the all-other-countries-but-not-East-Asian subgroup, the ANC-containing products had no apparent effect on LDL-C, HDL-C or FMD whereas in the studies conducted only in East Asian countries, the ANCs significantly reduced LDL-C (SDM: −0.45, p-value = 0.003, DM: −0.30 mmol/L, p-value = 0.000, n = 6), and increased HDL-C (SDM: +0.57, p-value = 0.000, DM: +0.15 mmol/L, p-value = 0.000, n = 6) and FMD (DM: +2.19%, p-value = 0.000, n = 2) (Supplementary Table S10a). Subgroup comparisons between studies conducted in East Asian countries and those carried out in the rest of the world confirmed a significant effectiveness in the upregulatory effects of the ANC-containing products in the Asian countries for HDL-C ((SDM): Q statistic = 5.21, p-value = 0.022; (DM): Q statistic = 5.51, p-value = 0.019) and for FMD ((SDM): Q statistic = 4.09, p-value = 0.043; (DM): Q statistic = 4.28, p-value = 0.038). For T-C and DBP, the evidence of a reduction by the ANC-containing products was stronger in the all-other-countries subgroup than in the East Asian ones. These results give some preliminary evidence of a potential influence of the East Asian countries associated characteristics on the effect of the ANC-containing products. On the other hand, the effects on SBP remained significant in both subgroups reinforcing a general effectivity of these type of compounds at regulating blood pressure. In support of this statement, when we classified the studies into those carried out in North America and those conducted in Europe (Supplementary Table S10b),

the ANCs still significantly reduced SBP and DBP in both subgroups. We additionally detected a significant reduction of T-C only in the European countries (SDM: −0.18, *p*-value = 0.017, *n* = 33; DM: −0.17 mmol/L, *p*-value = 0.000, *n* = 31) and some differences between the two subgroups at regulating the levels of insulin, with an apparent increase in North America studies and a reduction in European studies. Finally, stratification of the European countries into the Mediterranean and the Non-Mediterranean ones also shows some differences between the two areas, i.e., a significant reduction of LDL-C by the ANCs only in the Mediterranean countries (SDM: −0.35, *p*-value = 0.002, *n* = 14; DM: −0.19 mmol/L, *p*-value = 0.003, *n* = 14) or a significant increase in FMD only in the Non-Mediterranean area (SDM: +0.54, *p*-value = 0.006, *n* = 13; DM: +1.21%, *p*-value = 0.002, *n* = 13) (Supplementary Table S10c). SBP and DBP were downregulated in both areas of Europe.

2.4.4. Stratification by Specific Sources of ETs and ANCs.

Examination for specific differences between the main sources of compounds was investigated: pomegranate and nuts for ETs, and berries and red grapes/wine for ANCs. The complete results of this analysis can be seen in Supplementary Table S11. A summary with the most significant results and differences are listed in Table 4.

Comparison of studies by sources of ETs into pomegranate and nuts (Table 4a) demonstrated that nuts reduced significantly WC, T-C, LDL-C and TAGs. They also showed a tendency to reduce glucose levels. Further, nuts had a small but significant increase in HDL-C and a marginally significant increase in FMD. None of these effects were seen in the group of studies carried out with pomegranate. In addition, a very significant difference in the regulation of DBP was detected between these two types of products with studies conducted with pomegranate reporting that DBP was significantly reduced whereas nuts reported a small but significant increase in DBP (subgroups comparison (SDM): Q statistic = 12.95, *p*-value < 0.001; (DM): Q statistic = 17.32, *p*-value < 0.001).

Both sources of ANCs, berries and red wine/grapes, caused a significant reduction in blood pressure (Table 4b) but some differences were also detected between the two types of products. The berries reduced T-C and increased FMD whereas grapes and wine did not. In addition, the glycated hemoglobin was significantly reduced by the berries as opposed to the grapes/wine which increased the values of this biomarker (subgroups comparison (SDM): Q statistic = 8.59, *p*-value = 0.003; (DM): Q statistic = 9.41, *p*-value = 0.002).

Table 4. Comparative summary of the most significant effects on biomarkers of cardiometabolic risk of: (**a**) the ET-containing products after separation into the two main sources examined: pomegranate vs. nuts; (**b**) the ANC-containing products after stratification by the source of bioactive compounds: berries vs. red wine and red grapes.

(a)	**ET-Containing Products**								
Source	**Pomegranate**				**Nuts**				
	SDM (*p*-Value)	***n***	**DM (*p*-Value)**	***n***	**SDM (*p*-Value)**	***n***	**DM (*p*-Value)**	***n***	**Comparison between Subgroups (Q Statistic, *p*-Value)**
WC (cm)	−0.20 (NS)	1	−3.90 (NS)	1	−0.78 (0.027)	6	−1.51 (0.038)	5	SDM: 0.40, NS DM: 0.08, NS
T-C (mmol/L)	−0.04 (NS)	11	−0.02 (NS)	11	−0.32 (0.000)	17	−0.098 (0.000)	15	SDM: 10.83, 0.001 DM: 3.31, 0.069
LDL-C (mmol/L)	−0.07 (NS)	10	−0.05 (NS)	10	−0.26 (0.047)	16	−0.11 (0.000)	14	SDM: 1.31, NS DM: 0.66, NS
HDL-C (mmol/L)	+0.11 (NS)	10	+0.01 (NS)	10	+0.14 (NS)	13	+0.03 (0.029)	11	SDM: 0.09, NS DM: 0.30, NS
TAGs (mmol/L)	−0.05 (NS)	10	−0.01 (NS)	10	−0.33 (0.031)	16	−0.11 (0.000)	14	SDM: 1.44, NS DM: 1.10, NS
SBP (mm Hg)	−0.09 (NS)	8	−0.26 (NS)	6	−0.13 (NS)	13	−1.63 (NS)	9	SDM: 0.03, NS DM: 0.03, NS
DBP (mm Hg)	−0.46 (0.000)	8	−4.31 (0.000)	8	+0.06 (NS)	12	+0.58 (0.004)	10	SDM: 12.95, 0.000 DM: 17.32, 0.000
FMD (%)	+0.71 (NS)	1	+0.05 (NS)	1	+0.58 (0.058)	2	+1.04 (0.053)	2	SDM: 0.07, NS DM: 3.37, NS
Glucose (mmol/L)	−0.10 (NS)	7	−0.09 (NS)	7	−0.36 (0.079)	8	−0.14 (0.061)	8	SDM: 1.17, NS DM: 0.10, NS
(b)	**ANC-Containing Products**								
Source	**Berries**				**Red Wine/Red Grapes**				
	SDM (*p*-Value)	***n***	**DM (*p*-Value)**	***n***	**SDM (*p*-Value)**	***n***	**DM (*p*-Value)**	***n***	**Comparison between Subgroups (Q Statistic, *p*-Value)**
T-C (mmol/L)	−0.21 (0.021)	38	−0.16 (0.093)	35	−0.14 (NS)	44	−0.06 (NS)	43	SDM: 0.30, NS DM: 0.70, NS
SBP (mm Hg)	−0.25 (0.000)	38	−2.41 (0.000)	34	−0.21 (0.000)	36	−3.31 (0.014)	34	SDM: 0.11, NS DM: 0.35, NS
DBP (mm Hg)	−0.25 (0.001)	42	−1.57 (0.002)	37	−0.16 (0.000)	39	−1.50 (0.002)	35	SDM: 0.80, NS DM: 0.06, NS
FMD (%)	+0.46 (NS)	9	+1.39 (0.011)	8	−0.19 (NS)	10	−0.73 (NS)	10	SDM: 2.89, NS DM: 5.68, NS
Hb1Ac	−0.63 (0.044)	7	−0.20 (0.040)	6	+0.97 (0.038)	7	+0.26 (0.026)	7	SDM: 8.59, 0.003 DM: 9.41, 0.002

WC: Waist Circumference; T-C: Total Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; TAGs: Triglycerides; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; FMD: Flow Mediated Dilation; Hb1Ac: Glycated Haemoglobin; *n*: total number of studies included in the analysis; SDM: standardized difference in means (relative values); DM: Difference in means (units as indicated per each biomarker).

3. Discussion

As far as the authors are aware, this is the largest (128 reported dietary intervention studies involving 5438 participants from countries covering five continents) systematic review and subsequent meta-analysis investigating and comparing the effects of the consumption of plant food products and derived extracts containing substantial quantities of ANCs and (or) ETs, i.e., berries, red grapes and wine, pomegranate and nuts, on a number of well-established risk biomarkers associated with cardiometabolic disease. We have analyzed all these trials in an effort to determine the evidence accumulated so far in relation with their potential cardiometabolic benefits in humans, as well as the factors that cause variability in the results and may influence the response of the individuals to the consumption of these products. In the first part of our analysis, it was pertinent to investigate all these foods and food products together because some of them can contain high concentrations of both types of polyphenols, notably various berries [161,162]. This approach provides a most significant association between the intake of ANC- and (or) ET-containing products and beneficial changes in WC, T-C, SBP and DBP (reductions compared to control), or HDL-C (increase compared to control). Further, there were modest borderline significant reductions in fasting plasma glucose and TAGs and an increase in FMD. On the other hand, our analyses of data from studies separately investigating the effects of ET-rich foods (pomegranate and nuts) or ANC-rich foods and extracts (berries, red wine, red/black grapes) confirms effective and similar reduction of T-C levels by both types of products but points out differences between the beneficial effects of pomegranate and nuts (more efficacious at reducing WC, LDL-C, TAGs or glucose) and the benefits berries/grapes/wine (significant effective regulators of blood pressure). It is important to acknowledge that the beneficial effects detected by our analysis cannot be exclusively attributed to the ANCs or ETs present in them and that we cannot discard these effects may be also attributed to other components. Nevertheless, these results support the benefits of consuming food products containing ANCs and (or) ETs that, at least partially, may be due to these polyphenols.

With regards to the magnitude of the effects, it has been previously stated that a reduction of 1 mmol/L for non-HDL-C and an increase of 0.3–0.4 mmol/L for HDL-C are associated each with a one third reduction in ischemic heart disease risk [163] or a 22% reduction in coronary heart disease risk [164]. It has also been reported that a reduction of 12 mm Hg for SBP and of 5 mm Hg for DBP are accompanied by significant reductions of major cardiovascular events [165]. The results of our meta-analysis (expressed as DM values) show that, on average, the intake of ANC- and ET-containing products is associated with approximately 10-fold lower effects, i.e., a reduction of 0.10 mmol/L in T-C, an increase of 0.03 mmol/L in HDL-C and a decrease of 1.5–2.0 mm Hg for blood pressure, both SBP and DBP. These changes constitute between 1% and 3% change of the desirable threshold values for these biomarkers [164,166,167] and might be comparatively considered small or very small changes. This is also shown by the SDM values, mostly in the range of 0.1 to 0.2, generally considered as small changes [168]. Nevertheless, it is recognized that conventional risk factors interact to increase the risk for cardiometabolic diseases [169] and that combined treatment may be advantageous for the lowering of cardiometabolic events [170]. The fact that multiple vascular biomarkers that reflect multiple components of the cardiometabolic system are significantly altered in response to the consumption of ANC- and ET-rich foods and food products may contribute to the reduction of major cardiovascular events. In support of this, a previous meta-analysis of three prospective cohort studies concluded that ANCs intake was inversely associated with the risk of cardiovascular disease comparing the highest and lowest categories of intake [171]. Together, all these data suggest that foods and food products containing ANCs and ETs may act via the regulation of multiple biomarkers, including lipids, blood pressure and glucose homeostasis/insulin resistance.

The second aim of this systematic review and meta-analysis was to investigate the potential associations between some participant variables (baseline BMI, age, gender, smoking, geographical location where the study was carried out, health status and nature of the diet followed during the intervention) and the effects of the intervention with the food products containing ETs and (or) ANCs.

We show, for first time, that the significant effects of ET-rich products on T-C, LDL, TAGS and DBP and of ANC-rich food products on T-C, DBP and SBP were consistently observed in participants with BMIs $\geq$ 25 kg/m^2 (overweight/obese). Similar results were found in a previous meta-analysis, looking at the effects of flavanol-containing products in T-C [29]. Together, these results suggest that supplementation with polyphenol-rich products may have a beneficial impact on some cardiometabolic risk factors in overweight and/or obese people. In addition, significant effects of ET-rich products on SBP were observed in overweight/obese subjects but no significant effects on SBP were observed in the global study population, again supporting the need for population stratification, within such intervention studies, in order to discern effective regulation of biomarkers by the intake of bioactive compounds or products. It should be noted, however, that we found limited or non-evidence in normoweight participants since the number of studies reported was very small (n = 3). Thus, further investigation of the effects of ANC- and ET-products, and of other bioactive compounds, in individuals with BMI < 25 kg/m^2 are needed. With regard to the influence of participant sex, smoking habits, health status and habitual diet, there were, in general, a very small number of trials that provided this information in a useable form, limiting our ability to investigate how these host factors might affect the responses to intervention with foods and food products containing ANCs and/or ETs. Nevertheless, in the subsequent paragraphs we discuss some of the most relevant effects found in this meta-analysis for some of these factors.

Different responses were observed depending on the biomarkers analyzed and the health status of the participants. Accordingly, the beneficial effects of ANC-rich foods and extracts on blood pressure appeared to be independent of the participant health status, whereas we found some differences between healthy, 'at risk' and diseased participants in the effects of these foods on WC and T-C. On the other hand, supplementation with the ET-containing products modified WC, T-C and DBP only in healthy people, but affected FMD, LDL and TAGs in the 'at risk' and disease subpopulations. Previous reviews and meta-analyses had already indicated differences in the response to the intake of these and other bioactive compounds depending on the health status of the sample population. For example, systematic reviews of the effects of interventions with ANCs on lipid biomarkers have reported that only ANCs cause significant reductions in LDL-C in participants with hypercholesterolemia (4 out of 4 studies), but not in participants who had normal cholesterol (zero out of 8 studies) [172]. It was also reported that ANCs supplementation significantly decreased T-C, TAGs, LDL-C and increased HDL-C in dyslipidemic patients (6 studies including 586 subjects [173]. Similarly, it has been reported that high-dose quercetin supplementation caused a significant reduction in blood pressure in stage 1 hypertensive participants but was not affected in pre-hypertensive participants [174]. Also, the consumption of green tea [175,176], black tea [177], and flavanol-containing tea, cocoa and apple products [29] had beneficial effects on blood lipids both in healthy subjects and in patients with hyperlipidaemia or in individuals with cardiovascular risk and/or diagnosed diseases. On the contrary, meta-analyses conducted with flavonols [28], cocoa products [178], and soy products [179] reported beneficial effects in LDL-C, HDL-C and TAGs in participants with cardiovascular risk and/or diagnosed diseases, but no effect on healthy participants. Overall, these results show some evidence of the influence of the health status on the cardiometabolic response to the intake of bioactive compounds but this interaction is complex and far from understood. It is essential to continue the research to clarify the impact of the health/disease baseline conditions of the participants on the response to bioactive compounds intake.

Previous studies have indicated that the country or area of the world where the clinical study was conducted appears to have some influence on the results of these types of interventions with plant bioactive polyphenols. For example, specific differences between East Asian and Non-Asian countries or between Mediterranean and Non-Mediterranean countries for some lipid biomarkers in response to the intake of flavonol-containing products [28] or flavanol-containing products [29] have been reported. In the current meta-analysis, we have also identified a few differences in the effects of the ET- and ANC-containing foods for some of the biomarkers examined between specific

countries or geographical locations. In the case of products with ANCs, a significant reduction of LDL-C was seen only in East-Asian countries, but not in the rest of other countries; whereas the products containing ETs were efficient at reducing LDL-C in the subgroup of all the countries, but not in the East-Asian ones. The rationale for these potential differences and as to why a particular country or world area population may benefit best from the intake of these or other bioactive compounds is not yet known. In addition to the fact that the number of studies per subgroup of countries remains low and the results are very unstable, important country-associated features, such as the ethnicity of the participants, which may influence greatly the results have not been clearly indicated in most of the revised publications included in this meta-analysis and in previous ones [28,29]. Future studies should better describe these country-related characteristics of the participants.

Although the source of bioactive compounds cannot be categorized as a host determinant of interindividual variability, it has been already shown that it can significantly affect the response to the intake of these compounds [33]. In our meta-analysis we also stratifiyed the results taking into account the main categories of foods investigated, i.e., pomegranate, nuts, berries and red grape/wine. Our results highlighted differences in the effects between pomegranate and nuts. The pomegranate products significantly reduced DBP, but had no apparent effect on SBP, whereas nuts were found to cause a small but significant increase in DBP and had no significant effect on the SBP either. Also, nuts were associated with significant reductions in WC, T-C, LDL-C and TAGs, as well as a borderline increase in FMD, but these effects were not observed for the pomegranate. In comparison with our results, we found both agreement and disagreement with previous meta-analyses. For example, Sahebkar and colleagues reported significant reductions in SBP and DBP caused by the consumption of pomegranate products [24], but no significant effects on plasma lipids/lipoproteins [25]. Mohammadifard et al. [22] saw significant reductions in SBP only in participants without diabetes and significant reductions in DBP (but not SBP) in response to all nut supplementation and suggested that this was largely due to pistachios. Also, the effects of tree nuts were shown to reduce T-C, LDL-Cand TAGs [23]. Regarding the ANC-containing products, a previous meta-analysis looking at the supplementation with blueberries and their effect on blood pressure concluded that the results were not convincing and that more RCTs were needed [19]. In the current meta-analysis, ANC-containing products have come out as quite consistent regulators of SBP and DBP. Separation between studies conducted with berries and those carried out with red grapes/wine confirmed that both sources of ANCs significantly lowered SBP and DBP and supported that this type of bioactive compounds might have an impact on blood pressure regulation. We additionally found that the berries but not the grapes/wine significantly reduced T-C and increased FMD. In agreement with this last result, a recent meta-analysis on 24 RCTs showed that both acute and chronic supplementation with ANC-rich foods or extracts significantly improved FMD and improved wave velocity after acute consumption [21].

Various factors might be implicated in the differences found between the different types of food investigated. Most of the studies gathered in our meta-analysis, as well as in previous ones, have been conducted with various whole foods or derived extracts which are complex mixtures of compounds and thus, we cannot discard that the differences observed may be caused by differences in the types and doses of ETs or ANCs provided by these products and (or) by differences in the presence of other bioactive components with a beneficial effect. For example, there is a substantial literature describing the beneficial effects of nuts on biomarkers of cardiovascular health and on cardiovascular events see reviews by Schwingschackl et al., 2017 [180] and by Mayhew et al., 2016 [181], but it is not yet known whether these benefits may be due to the fatty acids/lipids, the polyphenols (which are mainly in the skins) or a combination of the two. Additional evidence from further well-designed trials are required before we can unequivocally attribute the benefits to the specific ETs or ANCs present in these products.

In addition to the factors examined in this meta-analysis, other important factors that may play a critical role in the inter-individual variability and can contribute to explain the lack of consistent evidence in humans of the beneficial cardiometabolic effects of the ANC- and ET-containing food

products (as well as of other polyphenol-containing foods and extracts) are: bioavailability of all these compounds and their derived metabolites [182,183], individuals enterotypes and functional stratification of the gut microbiome profile (i.e., metabotypes) [31,184,185], and the (epi)genetic characteristics of the host individual.

Regarding microbiota enterotypes, it is important to note that ETs are hydrolyzed into ellagic acid (EA) and further broken down into urolithin metabolites by the gut bacteria. Urolithins are much better absorbed and reach significant concentrations in plasma that can persist for hours in the human body after the intake of ETs-containing products suggesting that these urolithins may be the actual bioactive molecules [186]. Recently, it was reported that three urolithin metabotypes, based on the qualitative and quantitative proportions of urolithins produced, were consistently observed across multiple intervention studies and appeared to be independent of the ET food source and age or health status of participants [184]. These observations create a new paradigm where the urolithin metabotype of the participants should be determined and included as a covariate in future studies investigating the effects of consuming ET-rich products such as pomegranate and nuts. Along these lines, a recent RCT has shown that intervention with a purified pomegranate extract containing mainly ETs significantly improved lipid/lipoprotein profiles only in participants who produced a particular type of urolithins (around 30% of the total sample population), whereas no significant effects were detected when all participants were included in the analysis [31]. Therefore, since the urolithin metabotype of participants has not been reported or used to stratify participants for the vast majority of studies investigating the effects of ET-rich foods that were included in the current meta-analysis, it is not surprising that the meta-analysis failed to detect significant effects of ETs on lipoprotein profiles. Similar cases have been reported for the conversion of the soy isoflavone daidzein into equol, where volunteers can be categorized into equol producers and non-producers and this stratification might explain the discrepancy of the soy/isoflavones effects on human health, mainly cardiovascular. Thus, obesity has been correlated with the non equol-producer phenotype [187]. In addition, a positive correlation has been observed in the cardiovascular risk profiles and the equol-producer phenotype in pre-hypertensive postmenopausal women [188].

On the other hand, the relevance of the genotype-dependent response to dietary constituents is recognized as an additional key variability factor [189]. In this regard, an increasing number of genetic variants has been identified and related to obesity and diet-interaction and, the studied population has been segregated into groups of responders and non-responders in association with the specific genetic variations [190]. A number of studies have now also looked at the role of specific host genetic variants in response to the consumption of polyphenols or polyphenol-containing products, including genetic polymorphisms involved in: (i) the metabolism and transport of polyphenols such as Catechol-*O*-methyl transferase [191] or phase II enzymes UGT1A1 [192] and (ii) the cardiometabolic responses such as the lipid and blood pressure variation associated with the apolipoprotein e genotype in response to quercetin in overweight people [193] or the interaction between the *IL-6*-174 G/C polymorphism and the reduction of body fat following the intake of a polyphenol-rich apple juice [194]. More genes and polymorphisms involved in the response to polyphenols need to be identified and more RCTs need to be performed reporting and associating the presence of those relevant genetic polymorphisms, as well the microbiota composition and microbiota-derived metabolic phenotype with the differences in the response of the individuals to the consumption plant food bioactive compounds. These studies will contribute to better understanding of the effectiveness of these compounds in different subpopulations.

Some of the limitations of our meta-analysis are those inherent to this type of analysis. Given the heterogeneity of the RCTs included, the size of the sub-groups and the effect size detected for the variables investigated, some of the results presented here should be regarded with caution, even if some *p*-values resulted significant. A critical issue to consider would be the level of statistical significance accepted for the meta-analysis. We have accepted p-value < 0.05 as significant and indicated also some results that were marginally significant ($0.1 > p$-value > 0.05) since we thought that they could

be indicative of an effect (that, of course, would need future confirmation). Some researchers may believe that more restrictive p-values <0.01 or <0.001 should be applied [195] whereas, more recently, estimation based on effect size and confidence intervals is recommended [196]. In any case, the interpretation of the results of the meta-analysis may vary, especially for those most unstable results. Equally important is to address and understand the clinical relevance of the effects. We have addressed this in our discussion and suggested that, in general, the size of the effects of the dietary interventions with foods and (or) food products containing ANCs or ETs (or other polyphenols) may be considered small. Future RCTs should be sufficiently powered to validate these small changes. We additionally detected some publication bias for some of the variables investigated in this review. It is important that future publications of this kind of interventions also report the less favourable or negative results.

In conclusion, and despite these limitations, this is one of the largest meta-analysis performed in the area of the beneficial effects of the consumption of plant polyphenols in humans, and provides a good summary of the available information on the cardiometabolic effects of the intake of foods and food products containing ANCs and ETs. Overall, these foods and products appear to promote small but beneficial regulatory changes on a combination of risk factors and may contribute to prevent cardiometabolic diseases. Nevertheless, from a nutritional practice point of view, it is not yet possible to establish specific intake recommendations for these foods and (or) for the ANCs and ETs present in them since there are still some important challenges to solve. One of those is that there are still very few trials conducted with specific doses of purified ANCs or ETs compared with nutritionally matched placebos to demonstrate unequivocally the effects of these compounds and the doses needed. More RCTs designed for this purpose should be done in the future. Another important issue is that responses to polyphenol dietary interventions can be significantly dependent on different host factors and that within a study population there are subgroups of participants that respond strongly to a polyphenol intervention while other participants respond weakly or not at all. Our meta-analysis has explored some of the factors that might affect the response to the intake of ANC- or ET-containing products (baseline BMI, health status or food source) but our results were not sufficient to draw definitive conclusions in the subgroup analyses. Research to establish the determinants that cause inter-individual variability of the responses to the consumption of these and other bioactive compounds is a high current priority [33]. Ideally, better study design providing detailed descriptions, particularly around the choice and numbers of participants, should be addressed so that significant and clinically relevant effects on a primary outcome can be established for each subpopulation investigated with the ultimate goal of developing personalized nutrition strategies for human health and disease prevention. Such studies are still rare, and it is instead typical that stratifying of participants is done as an afterthought and was not considered in setting the participants' numbers at the stage calculating study power. An alternative approach would be to use data reported from completed and future trials to determine what factors affect responses to polyphenols. The ideal scenario would be the reporting of individual level data, notwithstanding the complex ethical and regulatory issues that this would raise. If all outcome responses were available for each participant along with pertinent participant characteristics such as age, gender, BMI, ethnicity, health status, habitual diet, smoking habit, baseline values for a series of risk biomarkers, relevant host genetic makeup and metabolic phenotype, this would allow studies to determine relationships between participant characteristics and their propensity to respond to polyphenols to advance rapidly. One could envisage an online repository for such information being of tremendous value and supporting high quality research that would relatively rapidly allow individual characteristics that determine responses to be identified. However, the ethical and regulatory issues are not trivial, and are not even consistent between territories, and such a data repository is not currently available.

4. Materials and Methods

A meta-analysis was performed to explore the potential regulatory effects of foods and (or) products containing ellagitannins (ETs) and/or anthocyanins (ACNs). We used the preferred reporting

items and statement guidelines for systematic review and meta-analysis protocols (PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [197], the Cochrane Handbook for Systematic Reviews of Interventions [198], and the Centre for Reviews and Dissemination's guidance for undertaking reviews in health care [199]. The protocol for this review was registered on the International Prospective Register of Systematic Reviews (PROSPERO) [200] with the registration number CRD42016037539.

4.1. Search Strategy and Study Selection

A comprehensive search on Medline [201] and on the Web of Knowledge [202] databases was conducted between April and June 2016. The search strategy included a combination of the following search terms: #1 AND #2 AND #3 AND #4 AND #5 (#1 polyphenol* OR ellagitannin* OR urolithin* OR hydrolizable tannin OR "ellagic acid" OR punicalagin OR peduncalagin OR sanguiin OR anthocyanin* OR anthocyanidin* OR pelargonidin OR delphinidin OR cyanidin OR petunidin OR peonidin OR malvidin; #2 berry OR berries OR "black currant" OR nuts OR walnut* OR "black carrot" OR "purple corn" OR pomegranate OR aronia OR wine OR grape*; #3 trial OR experiment OR study OR studies OR intervention; #4 human* OR subject* OR men OR male OR women OR female OR patient* OR volunteer* OR participant*; #5 FMD OR "flow-mediated dilation" OR "flow-mediated vasodilation" OR "flow-mediated vasodilatation" OR "endothelial function" OR "endothelial dysfunction" OR "blood pressure" OR hypertens* OR "arterial pressure" OR "pulse pressure" OR cholesterol OR LDL OR HDL OR BMI OR "body mass index" OR waist* OR HOMA-IR OR HOMA2 OR "homeostatic model assessment" OR insulin* OR QUICKI OR "impaired sensitivity" OR "Syndrome X" OR "Metabolic Syndrome X" OR glucose OR "blood glucose" OR glycemia OR "glycemic control" OR HbA1c OR "glycosylated haemoglobin" OR "glycated haemoglobin" or "haemoglobin A1c" OR "hemoglobin A, glycosylated" OR "euglycemic clamp" OR dyslipidemia* OR hyperlipidemia* OR hypertriglyceridemia* OR triglyceride* OR triacylglycer*).

The search terms were queried using the "topic" field in the WOS database; whereas for PubMed search we used the corresponding Mesh Terms, when available, and the presence of the keywords in the title or abstract of the papers using the tag [TIAB].

Two authors independently assessed all papers and a third author double-checked data selection to reach a consensus with the final selected studies. Studies included in the meta-analysis were limited to human RCTs testing the effect of ET- or ANC-containing foods or products, which had a control group receiving a placebo (group of participants who were exposed to a similar test product but without the ETs or ANCs) and measured one or more of the defined outcomes. Additional exclusion criteria were: case series, case reports, cohort studies, case-control studies, co-intervention, and cross-sectional studies, studies with multifactorial interventions (dietary or physical activity co-intervention), studies written in a non-European language and duplications.

4.2. Data Extraction

Data extraction was performed in duplicate by two authors, independently, and cross-checked by a third author using a standardized data extraction form. Extracted data included: (i) publication details (year of publication, name of first author, name and e-mail of corresponding author, clinical trial registration number (when available), country where the study was carried out); (ii) participants' characteristics (gender, age, ethnicity, health status, menopausal status, smoking habits, baseline BMI, use of medication); (iii) study setting (total number of participants included in the study and in the analysis, design (cross-over or parallel), washout duration, treatment duration, number of arms and description, number of participants located in each arm and completing the study, composition of test and placebo, dose and mode of administration); and (iv) information on reported outcomes (type of sample, changes in the outcome, values before and after intervention, p-value when available, dropouts). Before analysis, outcomes on blood lipid levels and glucose levels were converted to mmol/L if reported in a different unit.

4.3. Assessment of the Risk of Bias

A systematic assessment of the risk of bias for each of the included studies was based on the Cochrane Collaboration measurement with some modifications [198]. The specific items used for the assessments of each study are those used in a previous meta-analysis [28]: (1) selection bias—random sequence generation, allocation concealment (in each item, yes = 1; no = 0, unclear = 0); (2) performance bias—blinding (yes = 1 for each participants, researchers and statisticians, no = 0, unclear = 0), measurement of compliance (1 for biomarker measure, 0.5 if compliance information was collected by counting non used capsules or recipients, or by self-reporting, 0 if no measurement of compliance was done or the information is insufficient); (3) attrition bias – flow of participants (1 if flow of participants is explained in detail, including number of withdrawals and reasons, 0 if there is no information or insufficient information); (4) other bias—baseline comparability between test and control groups (yes = 1, no = 0, unclear = 0), data report (1 if pre and post data or change is reported in table with central measure and spread for placebo and treatment groups, and number per group, 0 if anything is missing), industry funding (0 if any commercial source provided some or all monetary funding for the trial, if a company carried out a study "in house", if any of the authors was employed by a relevant industry or if it was unclear that there was any kind of industry funding, 1 if there was no funding from industry or if the only involvement of a company was to provide any ingredient for the intervention). Studies were rated as low risk of bias when total score was $\geq$8 and $\leq$10, moderate risk of bias when total score was $\geq$5 and <8 and high risk of bias when total score was below 5.

4.4. Data Analysis

Data for each outcome were analyzed using the Comprehensive Meta-Analysis Software, version 3.0 (Biostat, Englewood, NJ, USA) [203]. Standardized difference in means (SDM), standard error (SE) and the 95% confidence intervals (CI) were calculated and pooled using random effects models to determine test/placebo differences across studies. We additionally determined absolute difference in means (DM) to estimate effect size. The heterogeneity of studies was assessed using the Cochran's Q statistic, the between-studies variance (T^2) and I^2 (the proportion of total variation contributed by between-study variability) where I^2 values equal to 25%, 50% and 75% were considered as low, moderate and high heterogeneity, respectively [204]. Publication bias was assessed visually with funnel plots and statistically by applying the Egger's regression test [205]. Further assessment of the possible associations between the overall effects of the ETs and/or ANCs supplementation and the duration of the intervention was examined using random-effects meta-regression analysis.

Quality of evidence was assessed based on the GRADE system [198]. Level of evidence was downgraded from high to moderate in the presence of serious risk of bias across studies or serious risk of reporting bias, and downgraded to low if both were present.

Subgroup analyses were conducted to explore potential factors that may introduce heterogeneity into the studies and influence the inter-individual variability in the response to supplementation with the ET- and/or ANC-containing products. We selected those factors that were investigated previously [28,29] and were most clearly reported in the selected articles (Table 5). Briefly, we included factors that might be attributed to some of the individuals' characteristics, such as baseline BMI, sex, smoking habits and medication/health status. Age or ethnicity could not be assessed due to unclear reporting. We also included stratification by the country in which the study was carried out, the source and form of administration of the ETs and/or ANCs, as well as the type of diet reported to be followed during the intervention. For each subgroup, the pooled effects (SDM and DM) and the significance of these values were estimated. Statistical comparisons between subgroups were performed by applying a random-effects analysis and calculation of the between-categories Q statistic and the corresponding p-values. A p-value <0.05 was statistically significant. Differences with a p-value <0.1 and $\geq$0.05 were reported as marginal.

Table 5. Potential factors influencing the heterogeneity in the responses to the supplementation with ellagitannins and/or anthocyanins-containing products investigated in this meta-analysis.

Factors					
Baseline BMI	25.0 [a] (normal and (or) underweight)			≥25.0 (overweight and (or) obese)	
Sex	Women			Men	
Smoking	Non-smokers			Smokers	
Country where the study was conducted	East Asian countries (Japan, Korea, China)	All-other-countries-but-not-East Asian	North America (USA, Canada)	European countries	
				Non-Mediterranean countries (Denmark, Norway, Finland, The Netherlands, Germany, Poland, UK, Scotland, France, Czech Republic)	Mediterranean countries (Italy, Spain, Greece)
Medication	Yes			No	
Health status	Healthy individuals [b]		Individuals 'at a risk' of disease [c]		Individuals with a reported disease [d]
Main source of compounds	Ellagitannins			Anthocyannins	
	Pomegranate		Nuts	Berries	Red wine and red grapes
Diet during intervention	Controlled diet (specifically indicated to have restriction for the consumption of polyphenols or plant foods)			Usual diet (no changes in the usual diet of the participants or NR)	

[a] BMI cut-off values as established by the WHO; [b] Includes individuals specifically reported as healthy and not medicated (in some cases medication was not reported, NR); [c] Includes individuals not medicated that were overweight and (or) obese, or specifically indicated to be borderline, mild condition or 'at risk' of a disease; [d] Includes individuals with one or more than one of the following disorders: dyslipidemia, glucose disorders or type-2 diabetes, blood pressure disorders (hypertension), medicated obesity, metabolic syndrome (most cases were also medicated but in some cases medication was NR).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

LDL-C	Low density lipoprotein cholesterol
HDL-C	High density lipoprotein cholesterol
ANCs	Anthocyanins
ETs	Ellagitannins
FW	Fresh weight
RCTs	Randomized-controlled trials
BMI	Body mass index
SDM	Standardized difference in means
DM	Difference in means
WC	Waist circumference
T-C	Total cholesterol
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
FMD	Flow mediated dilation
TAGs	Triglycerides
HbA1c	Glycated hemoglobin
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
WHO	World health organization
NR	Not reported
GRADE	Grading of Recommendations Assessment, Development and Evaluation

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Risk thresholds for alcohol consumption: combined analysis of individual-participant data for 599 912 current drinkers in 83 prospective studies

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Summary

Background Low-risk limits recommended for alcohol consumption vary substantially across different national guidelines. To define thresholds associated with lowest risk for all-cause mortality and cardiovascular disease, we studied individual-participant data from 599 912 current drinkers without previous cardiovascular disease.

Methods We did a combined analysis of individual-participant data from three large-scale data sources in 19 high-income countries (the Emerging Risk Factors Collaboration, EPIC-CVD, and the UK Biobank). We characterised dose–response associations and calculated hazard ratios (HRs) per 100 g per week of alcohol (12·5 units per week) across 83 prospective studies, adjusting at least for study or centre, age, sex, smoking, and diabetes. To be eligible for the analysis, participants had to have information recorded about their alcohol consumption amount and status (ie, non-drinker *vs* current drinker), plus age, sex, history of diabetes and smoking status, at least 1 year of follow-up after baseline, and no baseline history of cardiovascular disease. The main analyses focused on current drinkers, whose baseline alcohol consumption was categorised into eight predefined groups according to the amount in grams consumed per week. We assessed alcohol consumption in relation to all-cause mortality, total cardiovascular disease, and several cardiovascular disease subtypes. We corrected HRs for estimated long-term variability in alcohol consumption using 152 640 serial alcohol assessments obtained some years apart (median interval 5·6 years [5th–95th percentile 1·04–13·5]) from 71 011 participants from 37 studies.

Findings In the 599 912 current drinkers included in the analysis, we recorded 40 310 deaths and 39 018 incident cardiovascular disease events during 5·4 million person-years of follow-up. For all-cause mortality, we recorded a positive and curvilinear association with the level of alcohol consumption, with the minimum mortality risk around or below 100 g per week. Alcohol consumption was roughly linearly associated with a higher risk of stroke (HR per 100 g per week higher consumption 1·14, 95% CI, 1·10–1·17), coronary disease excluding myocardial infarction (1·06, 1·00–1·11), heart failure (1·09, 1·03–1·15), fatal hypertensive disease (1·24, 1·15–1·33); and fatal aortic aneurysm (1·15, 1·03–1·28). By contrast, increased alcohol consumption was log-linearly associated with a lower risk of myocardial infarction (HR 0·94, 0·91–0·97). In comparison to those who reported drinking >0–≤100 g per week, those who reported drinking >100–≤200 g per week, >200–≤350 g per week, or >350 g per week had lower life expectancy at age 40 years of approximately 6 months, 1–2 years, or 4–5 years, respectively.

Interpretation In current drinkers of alcohol in high-income countries, the threshold for lowest risk of all-cause mortality was about 100 g/week. For cardiovascular disease subtypes other than myocardial infarction, there were no clear risk thresholds below which lower alcohol consumption stopped being associated with lower disease risk. These data support limits for alcohol consumption that are lower than those recommended in most current guidelines.

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Introduction

Alcohol consumption guidelines vary substantially across the globe.[1,2] In the USA, for example, an upper limit of 196 g per week (about 11 standard UK glasses of wine or pints of beer per week) is recommended for men, and an upper limit of 98 g per week is recommended for women.[1] Similar recommendations apply in Canada and Sweden.[2] By contrast, guidelines in Italy, Portugal, and Spain recommend low-risk limits almost 50% higher than these.[1,2] At the other extreme, UK guidelines recommend low-risk limits for men almost half that recommended by US guidelines.[1,2]

Such variation in policy might reflect ambiguity about drinking risk thresholds associated with the lowest risk of mortality,[3–15] as well as uncertainty about the specific consequences of alcohol consumption, including those related to cardiovascular disease subtypes. For example, recent studies have challenged the concept that moderate alcohol consumption is universally associated with lower cardiovascular disease risk,[16,17] but the dose–response associations of alcohol consumption with cardiovascular disease subtypes remain poorly understood. Therefore, to help in the formulation of evidence-based alcohol policy, we analysed individual-participant data from 83 long-term prospective studies in 19 high-income countries. Our aim was to characterise risk thresholds for all-cause mortality and cardiovascular disease subtypes in current drinkers of alcohol.

Methods

Study design, data sources, and participants

We focused our study on current alcohol drinkers for three main reasons. First, alcohol guidelines provide recommendations about low-risk limits only for drinkers (we are unaware of any guidelines that encourage non-drinkers to consume alcohol). Second, a focus on current drinkers should limit potential biases that are difficult to control in observational studies (eg, reverse causality, residual confounding, and unmeasured effect modification) because ex-drinkers include people who might have abstained from alcohol owing to poor health itself,[18–20] as well as those who have changed their habits to achieve a healthier lifestyle. Third, never-drinkers might differ systematically from drinkers in ways that are difficult to measure, but which might be relevant to disease causation.[21]

We did a combined analysis of individual-participant data from three large-scale data sources available to our consortium, each constituting purpose-designed prospective cohort studies with quantitative information about alcohol consumption (appendix p 21). First, the Emerging Risk Factors Collaboration (ERFC) is a collaboration of prospective cohort studies with information about a variety of risk factors, cardiovascular disease outcomes, and mortality.[22] Of the 102 studies in the ERFC with information about alcohol status, 81 contained information about the quantity of consumption. Second,

Research in context

Evidence before this study

We searched for prospective epidemiological studies of alcohol consumption investigating disease risk thresholds published in any language up until March 1, 2017 (with no specified earliest date), in PubMed, Scientific Citation Index Expanded, and Embase using relevant terms ("alcohol", "mortality", "survival", "cardiovascular disease", "cohort", and "prospective"). We found many primary reports and literature-based reviews. However, no study had combined the following key features required to achieve reliable estimates of dose–response associations: availability of individual-participant data; quantitative assessment of alcohol consumption levels using validated instruments; periodic re-surveys of alcohol consumption levels; recording of large numbers of deaths (eg, >20 000 deaths); and sufficient detail and power to disaggregate incident cardiovascular disease outcomes into subtypes (eg, >20 000 incident total cardiovascular disease outcomes).

Added value of this study

The current study combined all the key study design features mentioned above, and afforded several additional advantages. First, it reduced the potentially distorting effects of reverse causality by focusing on current drinkers without previous cardiovascular disease who survived at least 12 months of follow-up. Second, it enhanced generalisability by including individual-participant data from 83 prospective studies in 19 different high-income countries. Third, it used a variety of established and emerging risk factors, enabling investigation of potential confounders and mediators.

Implications of all the available evidence

The chief implication of this study for public policy is to support reductions of alcohol consumption limits in existing guidelines, suggesting that the threshold for lowest risk for all-cause mortality is about 100 g per week (about 5–6 standard UK glasses of wine or pints of beer per week). The chief implication for scientific understanding is the strengthening of evidence that the association between alcohol consumption and total cardiovascular disease risk is actually comprised of several distinct and opposite dose–response curves rather than a single J-shaped association.

EPIC-CVD, a ten-country case-cohort study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) prospective cohort study, had quantitative alcohol information from 22 of its 23 contributing centres.[23] Third, UK Biobank—a single large prospective study—had cohort-wide data about quantitative alcohol consumption.[24] Therefore, our combined analysis included information from a total of 83 prospective studies that each used broadly similar methods to quantify alcohol consumption, record risk factors, and ascertain cause-specific death and cardiovascular disease events. We harmonised records of alcohol consumption across the contributing studies using a conversion of 1 unit=8 g of pure alcohol to a standard scale of grams per week (appendix pp 1–2), enabling a common analytical approach despite variation in the methods used (eg, self-administered *vs* interview-led questionnaires; food frequency questionnaires *vs* dietary recall surveys), and in consumption scales over different periods of ascertainment. Details of contributing studies are in appendix pp 3–4, 10–11.

To be eligible for the analysis, participants had to have information recorded about their alcohol consumption amount and status (ie, non-drinker *vs* current drinker), plus age, sex, history of diabetes and smoking status, at least 1 year of follow-up after baseline, and no known baseline history of cardiovascular disease (defined as coronary heart disease, other heart disease, stroke, transient ischaemic attack, peripheral arterial disease, or cardiovascular surgery); appendix p 21. The main analyses focused on current drinkers, whose baseline alcohol consumption was categorised into eight predefined groups according to the amount in grams consumed per week: >0–≤25, >25–≤50, >50–≤75, >75–≤100, >100–≤150, >150–≤250, >250–≤350, and >350 g per week. We assessed alcohol consumption in relation to all-cause mortality, total cardiovascular disease, and the following cardiovascular disease subtypes (defined in appendix p 5): fatal and non-fatal myocardial infarction; fatal and non-fatal coronary disease excluding myocardial infarction; fatal and non-fatal stroke (including ischaemic, haemorrhagic, subarachnoid, and unclassified subtypes of stroke); fatal and non-fatal heart failure; and mortality from other cardiovascular causes, including cardiac dysrhythmia, hypertensive disease, sudden death, and aortic aneurysm.[7,17,25] In analyses of cardiovascular disease subtypes, participants contributed follow-up time until the first outcome recorded (ie, cardiovascular deaths preceded by non-fatal outcomes were not included). Event times were censored at the end of follow-up or death from non-cardiovascular causes.

Statistical analysis

Hazard ratios (HRs) for alcohol consumption were calculated separately within each study using Cox regression models, stratified by sex and with adjustment for known confounders: age, smoking status (current *vs* non-current) and history of diabetes. To account for EPIC-CVD's case-cohort design (which was used because lipids and other cardiovascular disease biomarkers were measured only in the case-cohort subset and not the full EPIC cohort), the Cox models for cardiovascular disease events were adapted using Prentice weights and stratified by centre.[26] For the four case-control studies nested within prospective cohorts of the ERFC, odds ratios were calculated using, as appropriate, conditional or unconditional logistic regression models, taking into account relevant matching factors. Study-specific estimates were then pooled across studies by random-effects meta-analysis.[27] We tested for violation of the proportional hazards assumption by including time interactions with alcohol consumption. To avoid model overfitting, studies with fewer than five incident cases of a particular outcome were excluded from analyses of that particular outcome.

To correct for measurement error and within-person variability in alcohol consumption over time, we estimated long-term average (henceforth, "usual") alcohol consumption using multi-level regression calibration and information from 152 640 serial assessments in 71 011 individuals from 37 studies. This calculation was achieved either by regressing re-survey measurements (for the repeat alcohol assessments available in the ERFC studies and UK Biobank) or lifetime alcohol consumption measurements (for calculated lifetime alcohol consumption measurements available in EPIC-CVD) on baseline alcohol consumption, adjusted for duration of follow-up and baseline age, sex, smoking status, history of diabetes, other relevant covariate(s), and with random effects for study and re-survey.[28,29] The regression dilution ratio (ie, the calibration slope), which measures the extent of within-person variability,[28] was extracted from the calibration model. HRs in this paper relate to usual alcohol consumption levels unless specified otherwise.

We assessed the shapes of associations for all-cause mortality and cardiovascular disease outcomes by calculating study-specific HRs within the predefined groups of baseline alcohol consumption, pooled them by multivariate random-effects meta-analysis, and plotted them against mean usual (and baseline) alcohol consumption within each group. We estimated 95% CIs for each group (including the reference group) that corresponded to the amount of information underlying each group.[30,31] For each major outcome, we determined the best fitting first or second order fractional polynomial[32] to describe the association with baseline alcohol consumption (using a 1% significance level as evidence for a second order fractional polynomial over a first order fractional polynomial) using Cox regression models stratified by sex, study, and centre. Further analyses assumed a linear association with alcohol consumption, expressing results per 100 g per week (12·5 units/week) in usual alcohol consumption. To assess the effect of excluding known current drinkers with missing alcohol consumption data, we did a sensitivity analysis using multiple imputation within studies, before combining

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	ERFC	EPIC-CVD	UK Biobank	Participants with resurveys of alcohol consumption
Study level characteristics				
Location	81 studies in 19 countries	22 centres in 10 European countries	England, Scotland, and Wales	37 studies in 15 countries
Years of recruitment	1964–2008	1990–2002	2006–10	1964–2010
Year of most recent endpoint follow-up	2013	2009	2016	2016
Participant level characteristics				
Total participants	356 819	30 702	358 833	89 499
Known current drinkers at baseline	247 504	26 036	326 372	71 011
Weekly baseline alcohol consumption in current drinkers				
>0–≤25 g per week	53 418 (22%)	7906 (30%)	39 641 (12%)	12 301 (17% [11 g/week *vs* 36 g/week]‡)
>25–≤50 g per week	33 953 (14%)	3704 (14%)	39 334 (12%)	8365 (12% [38 g/week *vs* 56 g/week]‡)
>50–≤75 g per week	26 656 (11%)	2748 (11%)	42 907 (13%)	7322 (10% [63 g/week *vs* 80 g/week]‡)
>75–≤100 g per week	16 557 (7%)	2446 (9%)	36 780 (11%)	6394 (9% [87 g/week *vs* 98 g/week]‡)
>100–≤150 g per week	36 236 (15%)	2602 (10%)	55 815 (17%)	10 051 (14% [126 g/week *vs* 126 g/week]‡)
>150–≤250 g per week	31 645 (13%)	3090 (12%)	60 025 (18%)	12 255 (17% [193 g/week *vs* 173 g/week]‡)
>250–≤350 g per week	23 607 (10%)	1744 (7%)	26 669 (8%)	6927 (10% [303 g/week *vs* 248 g/week]‡)
≥350 g per week	25 432 (10%)	1796 (7%)	25 201 (8%)	7396 (10% [515 g/week *vs* 354 g/week]‡)
Baseline characteristics restricted to all current drinkers				
Alcohol consumption (g/week), median (5th–95th percentiles)	87·7 (2·2–522·4)	61·9 (2·6–404·0)	103·9 (11·8–420·8)	105·2 (6·0–482·8)
Age (years) at baseline	57·1 (8·7)	55·0 (9·2)	56·5 (8·0)	55·3 (8·2)
Sex				
Male	162 685 (66%)	13 508 (52%)	157 809 (48%)	44 360 (62%)
Female	84 819 (34%)	12 528 (48%)	168 563 (52%)	26 651 (38%)
Smoking status				
Not current	161 037 (65%)	17 608 (68%)	293 182 (90%)	50 930 (72%)
Current	86 467 (35%)	8428 (32%)	33 190 (10%)	20 081 (28%)
History of diabetes				
No	237 685 (96%)	24 875 (96%)	315 090 (97%)	68 159 (96%)
Yes	9819 (4%)	1161 (4%)	11 282 (3%)	2852 (4%)
BMI, kg/m²	26·1 (3·8)	26·4 (4·1)	27·0 (4·4)	26·1 (3·8)
HDL-C, mmol/L	1·40 (0·41)	1·40 (0·42)	Not available*	1·41 (0·41)
Total cholesterol, mmol/L	5·80 (1·17)	6·11 (1·16)	Not available*	5·78 (1·08)
Systolic blood pressure, mm Hg	136·5 (19·0)	138·4 (21·3)	137·9 (18·5)	134·6 (18·4)
Major outcomes restricted to current drinkers				
All-cause mortality events	32 813	784†	6720	6912
All cardiovascular disease	18 791	12 758	7469	11 597

Data are n, n (%), or mean (SD), unless otherwise indicated. ERFC=Emerging Risk Factors Collaboration. EPIC-CVD=European Prospective Investigation into Cancer and Nutrition—Cardiovascular Disease. BMI=body-mass index. HDL-C=high-density-lipoprotein cholesterol. *At the time of analysis, measurements of HDL-C and total cholesterol were not available in the UK Biobank. †All-cause mortality events from EPIC derive only from the 13 670 participants in the random sub-cohort of EPIC-CVD, rather than from the entire EPIC prospective study. ‡Mean consumption (g/week) at baseline *vs* resurvey.

Table 1: **Study-level and participant-level characteristics of the contributing data sources**

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the data in a meta-analysis. We investigated associations with alcohol type (wine, beer, and spirits), consumption frequency (dichotomised as drinkers who consumed alcohol on ≤2 days per week or those who consumed alcohol on >2 days per week) and episodic heavy drinking (dichotomised as binge drinkers who consumed ≥100 g per drinking occasion or non-binge drinkers who consumed <100 g per drinking occasion).

We used regression calibration methods similar to those described above to estimate and adjust for long-term levels of potential confounding factors or mediators in individuals with available information. HRs were adjusted for usual levels of available potential confounders or mediators, including body-mass index (BMI), systolic blood pressure, high-density-lipoprotein cholesterol (HDL-C), low-density-lipoprotein cholesterol (LDL-C), total cholesterol, fibrinogen, and baseline measures for smoking amount (in pack-years), level of education reached (no schooling or primary education only *vs* secondary education *vs* university), occupation (not working *vs* manual *vs* office *vs* other), self-reported physical activity level (inactive *vs* moderately inactive *vs* moderately active *vs* active), self-reported general health (scaled 0–1 where low scores indicate poorer health),

self-reported red meat consumption, and self-reported use of anti-hypertensive drugs. We investigated effect modification with formal tests for interaction, using a 0·1% significance threshold to make some allowance for multiple testing. Heterogeneity was investigated by grouping studies according to recorded characteristics and through meta-regression, assessed by the I^2 statistic.[33] Evidence of small study effects was assessed visually with funnel plots and by Begg and Mazumdar's test[34] and Egger's test.[35]

Methods we used to estimate reductions in life expectancy (years of life lost) are described in the appendix (pp 6–7). Briefly, estimates of cumulative survival from 40 years of age onwards in different categories of baseline alcohol consumption were calculated by applying estimated HRs (specific to age-at-risk) for cause-specific mortality to the detailed mortality component of the US Centers for Disease Control and Prevention's WONDER database,[36] which recorded 10 million deaths (from all causes) in more than 305 million individuals in the USA during 2007–10.[37,38] Results were modelled from age 40 years and enabled estimation of years of life lost between light drinkers (defined as those consuming >0–≤100 g/week of alcohol) and pre-defined groups of >100–≤200, >200–≤350, and >350 g per week. This method does not make use of the survival estimates from the modelled data; instead, it makes inferences by estimating age-at-risk specific HRs, which are then combined with external population age-specific mortality rates.[39]

Analyses used Stata (version 14.2 and 15.1). All p values presented are for 2-sided tests.

Role of the funding source

The funders of the study did not have any role in the study design, data analysis, or reporting of this manuscript. AMW and SK had full access to the combined dataset, and, together with EDA and JD, had responsibility for the decision to submit the manuscript for publication.

Results

Of the 786 787 participants with sufficient information for inclusion in this consortium, 186 875 (19%) reported not drinking at baseline, leaving 599 912 current drinkers without a history of cardiovascular disease at baseline who were eligible for the prespecified principal analysis. The current drinkers were derived from ERFC (247 504 participants), EPIC-CVD (26 036), and the UK Biobank (326 372; table 1). Baseline year of recruitment ranged from 1964 to 2010. The mean age of the participants was 57 years (SD 9). 265 910 (44%) of 599 912 participants were women, and 128 085 (21%) were current smokers (appendix p 12). About 50% reported drinking more than 100 g of alcohol per week, and 8·4% drank more than 350 g per week (table 1). During 5·4 million person-years (median 7·5 years of follow-up [5th–95th percentiles 5·0–18·4]), there were 40 310 deaths from all causes, (including 11 762 vascular and 15 150 neoplastic deaths), and 39 018 first incident cardiovascular disease outcomes, including 12 090 stroke events, 14 539 myocardial infarction events, 7990 coronary disease events excluding myocardial infarction, 2711 heart failure events, and 1121 deaths from other cardiovascular diseases (appendix p 13).

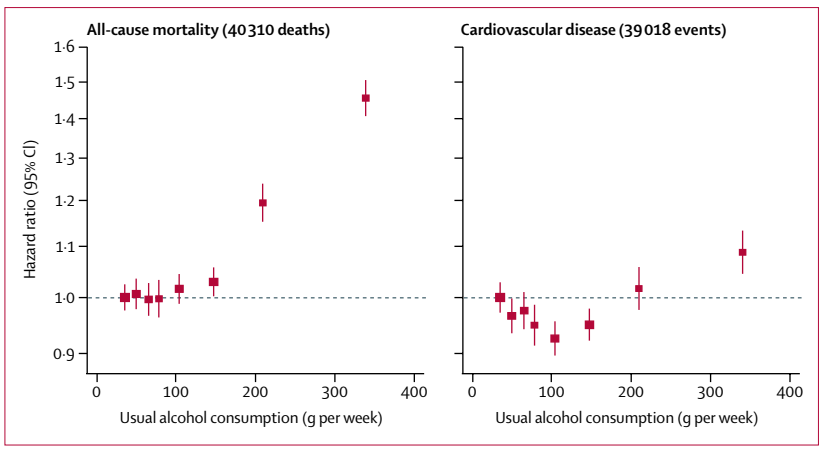

Figure 1: **Associations of usual alcohol consumption with all-cause mortality and the aggregate of cardiovascular disease in current drinkers**

Cardiovascular disease was defined as an aggregate of myocardial infarction, coronary heart disease, and stroke. Hazard ratios are adjusted for age, smoking, and history of diabetes, and stratified by sex and EPIC centre. The reference category is the lowest baseline alcohol consumption category (between 0 and 25 g/week). HRs are plotted against the mean usual alcohol consumption in each category. Sizes of the boxes are proportional to the inverse of the variance of the log-transformed hazard ratios. Vertical lines represent 95% CIs.

Baseline alcohol consumption varied substantially across studies, was generally lower in more recent calendar periods of recruitment, and was positively skewed (median 96 g/week [5th–95th percentiles 6–448]; appendix p 22). It was weakly and positively correlated with male sex, smoking status and amount, systolic blood pressure, HDL-C level, fibrinogen, and lower socioeconomic status (appendix pp 23–24). 152 640 serial assessments of alcohol consumption were available for 71 011 participants from 37 studies (median interval between baseline and serial measurements 5·6 years [5th–95th percentiles 1·04–13·5]). Participants with serial measurements were younger, had slightly higher baseline alcohol consumption, and were more likely to be men than those without serial measurements (table 1, appendix p 14). The regression dilution ratio for alcohol consumption was 0·50 (95% CI 0·47–0·52), similar to that for systolic blood pressure (0·52, 0·50–0·55) but lower than that for HDL-C concentration (0·74, 0·72–0·76) in a common set of participants.

For all-cause mortality, there was a positive and curvilinear association with alcohol consumption, with the lowest risk for those consuming below 100 g per week (figure 1, appendix p 25). Associations were similar for men and women (appendix p 26), but weaker at older ages (appendix p 27). There was a J-shaped association for the aggregate of cardiovascular disease outcomes (figure 1, appendix p 25). However, disaggregation showed two opposing sets of associations (figure 2).

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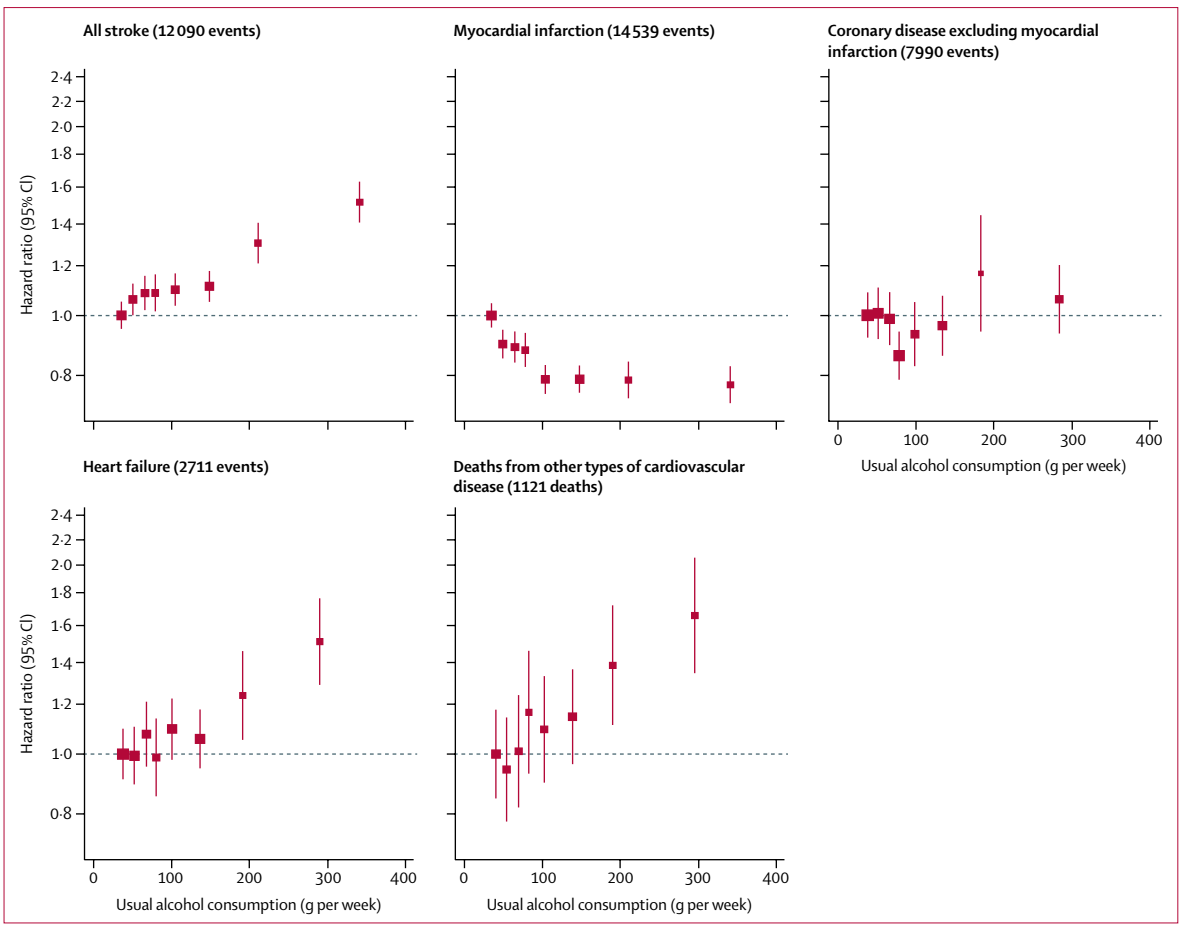

Figure 2: **Associations of usual alcohol consumption with cardiovascular subtypes in alcohol drinkers**
Hazard ratios are adjusted for age, smoking, and history of diabetes, and stratified by sex and EPIC centre. The reference category is the lowest baseline alcohol consumption category (between 0 and 25g/week). Hazard ratios are plotted against the mean usual alcohol consumption in each category. Studies with fewer than five events of any outcome were excluded from the analysis of that outcome. Sizes of the boxes are proportional to the inverse of the variance of the log-transformed hazard ratios. Vertical lines represent 95% CIs. Deaths from other cardiovascular disease include the following outcomes: cardiac dysrhythmia, hypertensive disease, sudden death, and aortic aneurysm.

After adjustment for age, sex, smoking, and history of diabetes, the amount of alcohol consumed had positive and roughly linear associations with stroke (HR per 100 g/week higher consumption 1·14, 1·10–1·17), coronary disease excluding myocardial infarction (1·06, 1·00–1·11), heart failure (1·09, 1·03–1·15), fatal hypertensive disease (1·24, 1·15–1·33), and fatal aortic aneurysm (1·15, 1·03–1·28; figures 2, 3). By contrast, there was an inverse and approximately log-linear association with myocardial infarction (0·94, 0·91–0·97; figures 2, 3). Stroke associations were similar for fatal and non-fatal outcomes (appendix p 28) and across subtypes (appendix p 29). However, for coronary disease excluding myocardial infarction, associations were stronger for fatal than non-fatal outcomes (appendix p 28). For myocardial infarction, inverse associations were possibly more pronounced with non-fatal than fatal outcomes (figure 3, appendix p 28).

With the following notable exceptions, further adjustment for additional covariates did not substantially change HRs (table 2, appendix pp 15, 30). First, adjustment for HDL-C level weakened the inverse association between alcohol consumption and myocardial infarction, but strengthened the positive association between alcohol consumption and both coronary disease and heart failure. Second, adjustment for systolic blood pressure strengthened the inverse association between alcohol consumption and myocardial infarction, but weakened the positive associations between alcohol consumption and all other cardiovascular disease outcomes. Our analysis confirmed the established association of alcohol consumption with cancers of the digestive system, which did not change after additional adjustment for the factors listed above (appendix p 16). Furthermore, additional adjustment for smoking amount abolished the apparent association of alcohol consumption with lung cancer (appendix pp 16), in line with the accepted view that alcohol consumption does not cause lung cancer.[40]

When including never-drinkers and ex-drinkers, we reproduced previously reported U-shaped associations of

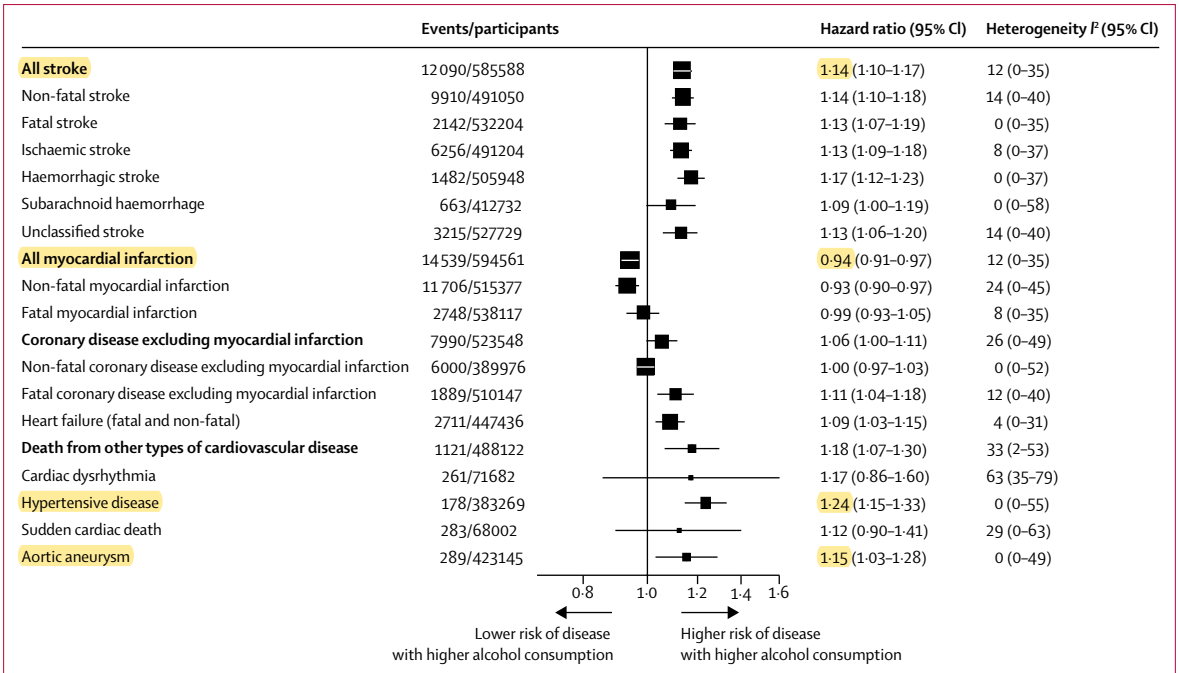

Figure 3: **Hazard ratios for subtypes of cardiovascular outcomes in current drinkers, per 100 g per week higher usual alcohol consumption**
Hazard ratios are adjusted for age, smoking, and history of diabetes, and stratified by sex and centre. Studies with fewer than five events of any outcome were excluded from the analysis of that outcome.

	All stroke	Myocardial infarction	Coronary disease excluding myocardial infarction	Heart failure	Deaths from other types of cardiovascular disease
Subset of participants with measurement of systolic blood pressure					
Cohorts/events	70/11 297	73/13 519	46/7789	39/2668	44/1019
Basic adjustment*	1·16 (1·11–1·22)	0·95 (0·91–0·99)	1·06 (1·00–1·12)	1·11 (1·04–1·18)	1·16 (1·06–1·27)
Plus adjustment for systolic blood pressure	1·10 (1·06–1·14)	0·91 (0·87–0·94)	1·03 (0·97–1·10)	1·08 (1·02–1·15)	1·14 (1·03–1·25)
Subset of participants with measurement of high-density-lipoprotein cholesterol					
Cohorts/events	56/7982	61/9911	36/3608	29/1886	34/690
Basic adjustment*	1·16 (1·10–1·23)	0·93 (0·88–0·97)	1·07 (0·98–1·17)	1·09 (1·00–1·19)	1·22 (1·06–1·40)
Plus adjustment for high-density-lipoprotein cholesterol	1·17 (1·11–1·22)	1·00 (0·96–1·04)	1·13 (1·05–1·22)	1·14 (1·01–1·27)	1·22 (1·08–1·38)
Subset of participants with measurement of body-mass index					
Cohorts/events	68/11 733	71/14 217	43/7761	36/2566	42/1035
Basic adjustment*	1·15 (1·10–1·19)	0·95 (0·91–0·98)	1·06 (1·02–1·12)	1·12 (1·04–1·20)	1·16 (1·06–1·27)
Plus adjustment for body-mass index	1·14 (1·10–1·18)	0·94 (0·91–0·97)	1·06 (1·01–1·12)	1·10 (1·03–1·16)	1·16 (1·06–1·27)

Data are hazard ratio (95% CI) per 100 g per week higher usual alcohol consumption, unless otherwise indicated. Analyses were restricted to individuals with basic adjustment variables plus the additional variable. Studies with fewer than five events were excluded from the analysis of each outcome. *Basic adjustment includes age, smoking, and history of diabetes, and stratification by sex and centre.

Table 2: **Hazard ratios for major cardiovascular outcomes in current drinkers, without and with adjustment for usual levels of systolic blood pressure, high-density-lipoprotein cholesterol, or body-mass index**

alcohol consumption with total cardiovascular disease and all-cause mortality (appendix p 31). However, we observed notable differences in baseline characteristics between never drinkers and current drinkers (eg, in relation to sex, ethnicity, smoking, and diabetes status; appendix p 12), supporting the validity of focusing on current drinkers in our main analysis. We recorded similar findings to those reported above in sensitivity analyses that involved the following approaches: used multiple imputation rather than complete-case analysis (appendix p 32); used fractional polynomials (appendix p 34); used a fixed-effect meta-analysis (appendix p 35); included studies that

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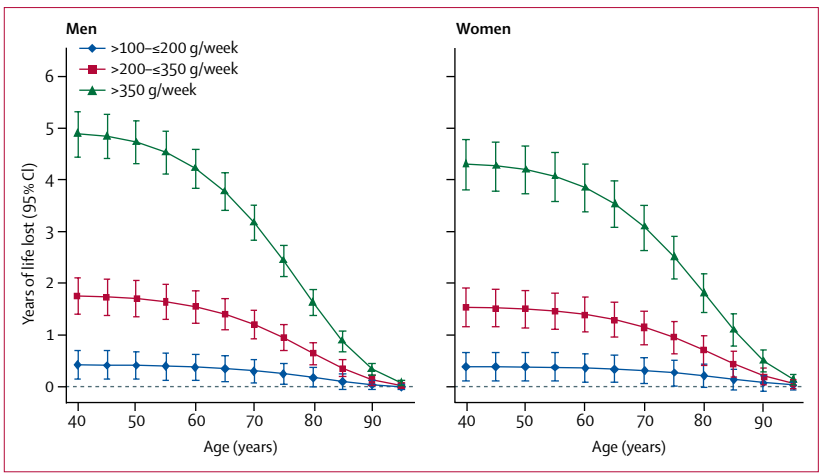

Figure 4: **Estimated future years of life lost by extent of reported baseline alcohol consumption compared with those who reported consuming >0–≤100 g per week**

The estimates of cumulative survival from 40 years of age onwards in the alcohol-drinking groups were calculated by applying hazard ratios (specific to age at risk) for all-cause mortality associated with categorised baseline alcohol consumption to US death rates at the age of 40 years or older. Mean usual levels of alcohol consumption within each baseline alcohol consumption category were 56, 123, 208 and 367 g per week, respectively, for the groups >0–≤100 g per week, >100–≤200 g per week, >200–≤350 g per week, and >350 g per week.

recorded fewer than five events for a particular outcome (appendix p 36); provided separate analyses of men and women (appendix p 17, appendix p 26); omitted outcomes recorded in the initial 5 years of follow-up (appendix p 18); excluded participants with diabetes or other known chronic diseases at baseline (appendix p 18); and restricted the analyses to studies that recorded both non-fatal and fatal endpoints (appendix p 37). Associations of baseline alcohol consumption with all-cause mortality were stronger in drinkers of beer or spirits than of wine, and in those drinking less frequently (when consuming the same weekly amount), including binge drinkers (appendix p 38). However, people showing these behaviours had higher baseline levels of smoking and other indicators of lower socioeconomic status, suggesting the potential for confounding effects (appendix pp 19–20). For cardiovascular disease subtypes, HRs tended to be higher in beer and spirit drinkers than in wine drinkers, but not significantly so in direct comparisons involving a common set of participants (appendix p 39).

We noted little heterogeneity in the studies contributing results for stroke (I^2=12%), myocardial infarction (I^2=12%), coronary disease excluding myocardial infarction (I^2=26%), heart failure (I^2=4%) or deaths from other types of cardiovascular disease (I^2=33%; figure 3). HRs for the cardiovascular disease outcomes we studied were broadly similar for different geographical regions, decade of study enrolment, by data source (ie, ERFC, EPIC-CVD, and UK Biobank), and alcohol assessment method (appendix pp 40–42). HRs for the cardiovascular disease outcomes were generally higher at younger ages, but did not vary substantially by sex, history of diabetes, proatherogenic lipids, BMI, smoking status, or other individual-level characteristics (appendix pp 43–45). There was no evidence of small study effects (appendix p 46). Our data showed no evidence of violation of the proportional hazards assumption.

In comparison to those who reported drinking >0–≤100 g (mean usual 56 g) alcohol per week, those who reported drinking >100–≤200 g (mean usual 123 g) per week, >200–≤350 g (mean usual 208 g) per week or >350 g (mean usual 367 g) per week had shorter life expectancy at age 40 years of approximately 6 months, 1–2 years, or 4–5 years respectively (figure 4). Similarly, men who reported consuming above the UK upper limit of 112 g per week had a shorter life expectancy at age 40 years of 1·6 years (95% CI 1·3–1·8), and men who reported drinking above the US upper limit of 196 g per week had a shorter life expectancy at age 40 years of 2·7 years (2·4–3·1) compared with men who reported drinking below these respective upper limits. Thus, men who reported drinking less than 100 g alcohol per week had about a 1–2 years longer life expectancy at age 40 years than those who reported drinking 196 g per week (appendix p 47). Women who reported drinking above either the UK threshold (112 g per week) or US threshold (98 g per week) had about 1·3 (1·1–1·5) years shorter life expectancy at age 40 years compared with women who reported drinking below these thresholds (appendix p 47). About 20% of the alcohol-related survival difference for men (and slightly less for women) was attributed to excess death from cardiovascular disease (appendix p 47). Similar findings to those for the US population were observed when modelling was based on EU mortality rates (data not shown).

Discussion

The main finding of this analysis was that the threshold for lowest risk for all-cause mortality was about 100 g per week. For men, we estimated that long-term reduction of alcohol consumption from 196 g per week (the upper limit recommended in US guidelines) to 100 g per week or below was associated with about 1–2 years of longer life expectancy at age 40 years. Exploratory analyses suggested that drinkers of beer or spirits, as well as binge drinkers, had the highest risk for all-cause mortality.

Our study has highlighted the complex and diverse potential mechanisms by which alcohol consumption may exert cardiovascular effects.[41,42] It has shown that the association between alcohol consumption and total cardiovascular disease risk comprises several distinct and opposite dose–response curves, rather than a single J-shaped association. In particular, whereas higher alcohol consumption was roughly linearly associated with a higher risk of all stroke subtypes, coronary disease excluding myocardial infarction, heart failure, and several less common cardiovascular disease subtypes, it was approximately log-linearly associated with a lower risk of myocardial infarction. Our results are concordant with recent observational data and Mendelian randomisation studies.[16,43–46]

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Our results contribute toward understanding of the basis for these directionally divergent cardiovascular disease associations. For example, our data have suggested that elevated systolic blood pressure could mediate alcohol consumption's positive association with stroke and coronary disease excluding myocardial infarction.[44,47,48] By contrast, pathways related to HDL-C (but not necessarily HDL-C itself[49–52]) could mediate alcohol consumption's inverse association with myocardial infarction. Both blood pressure and HDL-C are known to increase in response to alcohol consumption.[50] They have contrasting associations with cardiovascular disease outcomes: the inverse association of HDL-C with cardiovascular disease is substantially stronger for coronary disease than stroke,[53,54] whereas the positive association of systolic blood with cardiovascular disease is considerably stronger for stroke than coronary disease.[55] However, we did not find convincing evidence that other known risk factors were important mediators or confounders.

Our study's access to individual-participant data avoided limitations of previous literature-based reviews.[56] To limit reverse causality, our study focused on current drinkers without baseline cardiovascular disease and omitted the initial period of follow-up. To limit confounding, our study adjusted for a variety of risk factors. To correct for misclassification in alcohol consumption and covariates, our study also used extensive information on serial assessments. Our results were robust to a variety of sensitivity analyses. Generalisability of the findings was enhanced by inclusion of data from 83 prospective studies based in many different high-income countries recruited between 1964 and 2010. Although alcohol consumption levels declined during this period, HRs were similar over calendar time.

Nevertheless, our study has some potential limitations. Self-reported alcohol consumption data are prone to bias and are challenging to harmonise across studies conducted over different time periods that used varying instruments and methods to record such data.[20,57] We did not, however, identify major differences in results across studies that used differing alcohol measurement instruments. Despite our study's access to extensive serial alcohol re-surveys from mid-life, our study could not investigate alcohol consumption during the entire life course. Misclassification in outcomes would have diluted dose-response associations, suggesting that true underlying associations of alcohol consumption with cardiovascular disease subtypes are stronger and more divergent than we observed. Because we did not generally have access to additional alcohol-related adverse outcomes (eg, non-fatal liver disease, injuries, or psychiatric comorbidities), we probably under-estimated potential benefits associated with lowering alcohol consumption. Because some individuals who reduced, but did not cease, alcohol consumption due to health complications were probably included in our analysis, we cannot exclude the effects of reverse causation (especially since some contributing studies did not record baseline chronic disease other than cardiovascular disease). Therefore, alternative study designs including randomised trials[58] are needed, to control more completely for residual biases (including those related to studying ex-drinkers and never-drinkers).

In conclusion, our study shows that among current drinkers, the threshold for lowest risk of all-cause mortality was about 100 g per week. For cardiovascular disease subtypes other than myocardial infarction, there were no clear thresholds below which lower alcohol consumption stopped being associated with a lower disease risk. These data support adoption of lower limits of alcohol consumption than are recommended in most current guidelines.

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See Online for appendix

Contributors

All the authors contributed to data collection, and to the design, analysis, interpretation, and re-drafting of this report. AMW and SK had full access to the combined data and did the statistical analysis. AMW, EDA, and JD drafted the manuscript and had responsibility for submission of the manuscript for publication.

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Declaration of interests

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For the **study website of the funding agencies that have supported individual EPIC centres** see http://www.phpc.cam.ac.uk/ceu/epic_cvd/funding-sources/

For the **study website of funding agencies that have supported individual cohorts of the ERFC** see http://www.phpc.cam.ac.uk/ceu/erfc/list-of-studies/

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Daily Drinking Is Associated with Increased Mortality

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Background: There is evidence that low-level alcohol use, drinking 1 to 2 drinks on occasion, is protective for cardiovascular disease, but increases the risk of cancer. Synthesizing the overall impact of low-level alcohol use on health is therefore complex. The objective of this paper was to examine the association between frequency of low-level drinking and mortality.

Methods: Two data sets with self-reported alcohol use and mortality follow-up were analyzed: 340,668 individuals from the National Health Interview Survey (NHIS) and 93,653 individuals from the Veterans Health Administration (VA) outpatient medical records. Survival analyses were conducted to evaluate the association between low-level drinking frequency and mortality.

Results: The minimum risk drinking frequency among those who drink 1 to 2 drinks per occasion was found to be 3.2 times weekly in the NHIS data, based on a continuous measure of drinking frequency, and 2 to 3 times weekly in the VA data. Relative to these individuals with minimum risk, individuals who drink 7 times weekly had an adjusted hazard ratio (HR) of all-cause mortality of 1.23 ($p < 0.0001$) in the NHIS data, and individuals who drink 4 to 7 times weekly in the VA data also had an adjusted HR of 1.23 ($p = 0.01$). Secondary analyses in the NHIS data showed that the minimum risk was drinking 4 times weekly for cardiovascular mortality, and drinking monthly or less for cancer mortality. The associations were consistent in stratified analyses of men, women, and never smokers.

Conclusions: The minimum risk of low-level drinking frequency for all-cause mortality appears to be approximately 3 occasions weekly. The robustness of this finding is highlighted in 2 distinctly different data sets: a large epidemiological data set and a data set of veterans sampled from an outpatient clinic. Daily drinking, even at low levels, is detrimental to one's health.

Key Words: Alcohol Use, Mortality, Cancer Mortality, Cardiovascular Mortality.

EXCESSIVE ALCOHOL USE accounts for 9.8% of deaths among working-age adults in the United States and continues to be a leading cause of premature mortality (Stahre et al., 2014). The threshold for safe levels of alcohol use has been discussed since the mid-19th century (Anstie, 1870). Currently, the National Institute on Alcohol Abuse and Alcoholism and the Centers for Disease Control and Prevention set the threshold at up to 1 drink daily for women and up to 2 drinks daily for men as within the U.S. dietary guidelines (Division of Population Health, 2016; National Institute on Alcohol Abuse and Alcoholism, 2016; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Because of the complexity of drinking behavior itself, defining these guidelines is difficult, and therefore, these recommendations have evolved over time (Stockwell and Room, 2012). Research on multiple components of drinking behavior, including number of drinking days per week, average drinks per day, and binge drinking, has informed the development of these recommendations.

There is an expansive body of literature on the relationship between alcohol use and health. Investigators have examined the impact of alcohol use on health conditions such as bodyweight, blood pressure, and stroke, as well as diseases including diabetes and multiple types of cancer (Foster and Marriott, 2006). The association between alcohol use and cardiovascular health has been researched extensively, with many studies finding evidence of a J-shaped curve (Costanzo et al., 2010a,b): Low-level drinkers, defined as drinking 1 to 2 drinks per day, have a reduced risk for cardiovascular mortality compared to either abstainers or heavier drinkers.

A prospective study conducted by Mukamal and colleagues (2003) examined alcohol consumption and the risk of myocardial infarction among men over a 12-year period in the Health Professionals Follow-up Study. Both quantity and frequency of drinking were analyzed, along with type of alcohol consumed and changes in consumption over time. Alcohol consumption was associated with decreased risk of myocardial infarction, regardless of alcohol type, and those

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who reported drinking 3 to 4 days per week or 5 to 7 days per week had decreased risk compared to those who drank less than once a week. Several other studies investigating alcohol use and cardiovascular health outcomes have also reported a protective effect for low-level drinking as measured by quantity and frequency in both men and women (Britton and Marmot, 2004; Mukamal et al., 2005, 2010; Tolstrup et al., 2006), and this protective effect has been corroborated by meta-analyses (Costanzo et al., 2010a,b; Larsson et al., 2015; Mostofsky et al., 2016; Ronksley et al., 2011).

More recently, however, several studies have challenged the benefits of alcohol for cardiovascular health. A prospective study of nearly 600,000 drinkers showed that for cardiovascular disease subtypes other than myocardial infarction, lower alcohol consumption was associated with lower risk of disease (Wood et al., 2018). Analysis of individuals from this cohort without baseline cardiovascular disease found that alcohol intake was associated with increased risk of nonfatal coronary heart disease, but associated with decreased risk of stroke (Ricci et al., 2018). In a Mendelian randomization study by Holmes and colleagues (2014), individuals with decreased genetic risk for consuming alcohol and alcohol use disorder, based on the presence of a genetic variant that alters alcohol metabolism, were compared to individuals with typical genetic risk. The authors found that individuals with the genetic variant associated with lower alcohol consumption had a more favorable cardiovascular profile and reduced risk of coronary heart disease than those without the genetic variant. This finding supports the beneficial effect of reduced alcohol consumption for cardiovascular health.

In contrast to the inconsistent findings regarding alcohol use and cardiovascular health, there is a consensus that alcohol use is associated with increased cancer incidence (Cao et al., 2015; Chen et al., 2011; Trichopoulou et al., 2010; World Cancer Research Fund and American Institute for Cancer Research 2018). Because of the strong association between cigarette smoking and cancer, and the confounding between smoking and alcohol use, teasing apart the relationship between low-level alcohol use and cancer has been particularly difficult. A recent study of 2 large U.S. cohorts found an association between low-level drinking (1 to 2 drinks/d) with minimally increased risk of any type of cancer (excluding nonadvanced prostate cancer) in both men and women, regardless of smoking history (Cao et al., 2015).

Investigators have also extended the examination of the association between alcohol use and all-cause mortality. Historically, studies of all-cause mortality have replicated the J-shaped curve evident with cardiovascular outcomes (Costanzo et al., 2010a,b; Di Castelnuovo et al., 2006; Klatsky and Udaltsova, 2007; Ronksley et al., 2011; Xi et al., 2017), but recent studies using updated methodologies and data have found no evidence for a protective effect of low-level drinking on all-cause mortality (GBD 2016 Alcohol Collaborators 2018; Goulden, 2016; Stockwell et al., 2016; Wood et al., 2018).

To further complicate matters, gender and race differences have also been reported. Sempos and colleagues (2003) analyzed a sample of African Americans who were followed for 19 years as part of the National Health and Nutritional Examination Survey Epidemiologic Follow-Up Study and found no protective effect for low-level drinking on all-cause mortality. Likewise, Kerr and colleagues (2011) found no protective effect of low-level drinking on all-cause mortality for African Americans when analyzing data from the National Alcohol Surveys, but a protective effect was found for Whites in the same sample. With respect to gender, Klatsky and Udaltsova (2007) reported that the protective effect of low-level drinking on all-cause mortality was stronger in women compared to men, whereas a recent meta-analysis found no protective effect at all of low-level drinking for women (Zheng et al., 2015).

In synthesizing these studies, it has been argued that protective effects of alcohol on health have been overestimated and that the association, instead of being causal, is due to the methodological limitations of observational studies. Chikritzhs and colleagues (2015) contend that no protective effects should be assumed in future estimates of alcohol-related burden of disease or in national drinking guidelines. Several areas of potential bias have been identified that could lead to spurious associations, including misclassifying former drinkers as abstainers, residual or unmeasured confounding, and selection biases (Chikritzhs et al., 2009; Fillmore et al., 2007; Goulden, 2016; Klatsky and Udaltsova, 2013; Naimi et al., 2005, 2017a,b). Whether moderate alcohol consumption confers health benefits to current drinkers continues to be a topic of strong debate in the scientific literature (Britton and Bell, 2017; Chikritzhs et al., 2015; Mukamal et al., 2016; Naimi et al., 2017a,b; Rabin, 2018).

With the advent of large-scale databases, a more thorough examination of alcohol consumption and mortality can be conducted. This current study analyzes the association between alcohol use patterns (quantity and frequency) and risk for mortality by using 2 large data sets to determine the nadir of risk: the quantity and frequency of alcohol use associated with the lowest mortality and to identify where risk begins to increase.

MATERIALS AND METHODS

Two large data sets were used in this study: the National Health Interview Survey (NHIS) and outpatient medical records from Veterans Health Administration (VA) clinics. Because the structure of the 2 data sets differs, the data and analysis are described individually for each data set.

NHIS

Data. The NHIS is an ongoing annual survey, representative of the civilian, noninstitutionalized, household population of the United States (Minnesota Population Center and State Health Access Data Assistance Center, 2016). Data from the 1997 to 2009 administrations of the NHIS were merged to examine the relationship of drinking pattern variables with mortality data (https://ihis.ipums.

org/ihis/). NHIS data were collected via computer-assisted personal interviews administered by interviewers employed and trained by the U.S. Census Bureau. Included in these analyses were 340,668 individuals aged 18 to 85 who were followed for at least 2 years (i.e., did not die in the first 2 years) and had mortality follow-up in the fourth quarter of 2011 based on linkage constructed by the National Center for Health Statistics between surveyed individuals and death certificate records from the National Death Index (National Center for Health Statistics, 2015). All individuals included were surveyed only once.

Coding of Alcohol Use. Since 1997, the NHIS has included 3 questions to assess typical quantity of drinking, frequency of drinking, and binge drinking over the past year, as well as 2 questions about lifetime alcohol use (Box 1). Respondents are instructed to include all types of alcoholic beverages, including liquor such as whiskey or gin, beer, wine, and wine coolers. The definition of a standard drink was not provided.

Never drinkers were defined as those who had never had 12 drinks or more in their lifetime. Former drinkers were defined as those who had 12 drinks or more in their lifetime, but had not consumed alcohol in the past year. Current drinkers were defined as those who had consumed alcohol on at least 1 day in the past year. Annual drinking frequency was converted to weekly drinking frequency by dividing by 52. For the purposes of this study, we defined binge drinking as consuming 5 or more drinks on an occasion for both men and women. We dropped 990 individuals (0.3% of total) from the analysis due to missing alcohol-related data.

Outcomes. The primary outcome analyzed was all-cause mortality. For secondary analyses, cancer mortality and cardiovascular mortality were evaluated using the available causes of death. Cancer mortality was coded by the NHIS, and we defined cardiovascular mortality as mortality due to either (i) diseases of the heart or (ii) cerebrovascular diseases.

Covariates. All analyses included self-reported gender, race, age at time of survey in 5-year bins, region of country, survey administration year, health and wellness factors, and socioeconomic factors. Health and wellness factors included current smoking status (never smoker, former smoker, and current smoker categorized into cigarettes smoked per day [CPD] ≤10, CPD 11 to 20, CPD 21 to 30, CPD >30), perceived health status (on a 4-level scale), exercise level (quartiles), and medical comorbidities (indicator variables for self-reported history of each of the following: AIDS, cancer, chronic obstructive pulmonary disease [COPD], diabetes mellitus, heart failure, liver disease, myocardial infarction, peripheral vascular disease, peptic ulcer disease, and rheumatoid arthritis). These comorbidities were chosen based on their inclusion in a clinical comorbidity index (Deyo et al., 1992). Socioeconomic factors included educational category (no degree, high school degree, some college, bachelor's degree or higher), current employment status (never worked, worked in the last week, did not work in the past 12 months, did not work in the last week but worked in the past 12 months), and whether the household received food stamps in the last calendar year.

Data Analysis. Proportional hazard analyses were completed using SAS 9.4 (SAS Institute Inc., (c) 2000-2008) PROC SURVEYPHREG using the NHIS adult-sample weight from the pooled surveys adjusted for ineligible respondents. Survey adjustments include design, cluster, primary sampling units, ratio nonresponse, and poststratification adjustments for sample adults. The outcome variable was age at either death or the fourth quarter of 2011 (time of censoring). In addition to the drinking pattern variables (current nondrinker, quantity, nonbinge frequency, and binge frequency), models included gender, race, birth cohort (classified as a categorical variable corresponding to 5-year bins), year of survey (as a fixed effect), exercise, comorbidity, and socioeconomic factors listed above.

Because prior literature indicated that the association between alcohol use patterns and morbidity/mortality may be different for men and women (Klatsky and Udaltsova, 2007), secondary analyses included stratification by gender. To minimize the effect of comorbid smoking on mortality, secondary analyses were run on the subset of individuals who were never smokers, defined by smoking fewer than 100 cigarettes in their lifetime. Secondary analyses stratified by gender and smoking status were run by using the domain statement in PROC SURVEYPHREG. Finally, to ensure observed associations were not due to survey year, we ran stratified analyses for each survey year and meta-analyzed the results.

Box 1 Alcohol Surveys

NHIS Alcohol Survey

1. In any 1 year, have you had at least 12 drinks of any type of alcoholic beverage?
2. In your entire life have you had at least 12 drinks of any type of alcoholic beverage?
3. In the past year, how often did you drink any type of alcoholic beverage? (0 to 365 days)
4. In the past year, on those days that you drank alcoholic beverages, on the average, how many drinks did you have? (1 to 90 drinks)
5. In the past year, how many days did you have 5 or more drinks of any alcoholic beverage (0 to 365 days)

VA Alcohol Survey (AUDIT-C)

1. How often did you have a drink containing alcohol in the past year? (never, ≤ monthly, 2 to 4×/month, 2 to 3×/wk, ≥4×/wk)
2. How many standard drinks containing alcohol did you have on a typical day when you were drinking in the past year? (1 to 2, 3 to 4, 5 to 6, 7 to 9, ≥10)
3. How often did you have 6 or more drinks on 1 occasion in the past year? (never, < monthly, monthly, weekly, daily, or almost daily)

VA

Data. Outpatient medical records were extracted from the VA Corporate Data Warehouse through the VA Informatics and Computing Infrastructure. Data were extracted from the Alcohol Use Disorders Identification Test-Consumption, a brief validated screen for alcohol use disorders consisting of 3 items to assess typical quantity of drinking and frequency of drinking and binge drinking over the past year (Box 1) (Bradley et al., 2003, 2007; Bush et al., 1998; Frank et al., 2008). The definition of a standard drink was not specified.

Inclusion criteria were date of birth between January 1, 1948, and December 31, 1968, and completing an alcohol survey in 2008 (the first full year after the survey was introduced). This resulted in a total sample size of 93,653. If a veteran completed more than 1 alcohol survey in 2008, the first administration was used for analysis.

Coding of Alcohol Use. Patterns of alcohol use were separated into drinking quantity when not binge drinking, frequency of nonbinge drinking, and frequency of binge drinking. Drinking quantity on typical drinking days was categorized into 3 levels: 1 to 2 (low-level drinking), 3 to 4, and 5 or more (binge drinking). Overall drinking frequency (binge drinking and nonbinge drinking) and binge drinking frequency are defined in the assessment. The frequency of nonbinge drinking was computed by taking the overall drinking frequency and subtracting the binge drinking frequency. Nonbinge drinking was further categorized into 5 levels: none, monthly or less, 2 to 4 times monthly (reference category), 2 to 3 times weekly, and 4 or more times weekly (see Table S1). Binge drinking frequency was categorized into 4 levels: none, monthly or less, 2 to 3 times monthly, and weekly or more.

Outcomes. All-cause mortality was censored on June 30, 2016. We were not able to examine cardiovascular mortality or cancer mortality because cause of death was not available.

Covariates. Covariates included age, gender, race, and comorbidity. Comorbidity was coded as individual indicator variables for ICD-9 diagnoses corresponding to AIDS, COPD, dementia, hypertension, diabetes mellitus, liver disease, peripheral vascular disease, rheumatic disease, and peptic ulcer disease. These comorbidities were chosen based on their inclusion in a clinical comorbidity index (Deyo et al., 1992). Smoking behavior, educational attainment, and employment status were not available in this database.

The sample was divided into current nondrinkers (those who reported never having an alcoholic drink in the past year) and current drinkers (those who reported having an alcoholic drink in the past year). No information was available regarding drinking history prior to the past year. Thus, we were not able to distinguish lifetime never drinkers from former drinkers.

Data Analysis. Proportional hazard analyses were completed using SAS 9.4. The outcome variable was age at either death or June 30, 2016 (date of censoring). In addition to the drinking pattern variables (current nondrinker, quantity, nonbinge frequency, and binge frequency), models included gender, race, birth cohort (classified as a categorical variable corresponding to 5-year bins), and comorbidity.

Table 1. Characteristics of Samples

	NHIS	VA
N	340,668	93,653
Sample type	Epidemiological	Outpatient clinic
Year of survey	1997 to 2009	2008
Average age at survey (SD)	47 (11.2)	53 (5.1)
Average number of years observed among nondeceased individuals (SD)	10.4 (3.8)	7.7 (0.22)
Person-years followed***	2,156,198	704,354
Mortality rate (deaths per 1,000 person-years)***	5	13
Women***	55%	11%
Men***	45%	89%
African American, non-Hispanic***	14%	27%
European American, non-Hispanic***	66%	52%
Hispanic***	16%	5%
Other race***	4%	16%
Current drinkers***	64%	45%
Current nondrinkers***	36%	55%
Never smokers	56%	N/A
Current smokers	22%	N/A
Former smokers	22%	N/A
Presence of significant medical comorbidity***	49%	54%

N/A, not available; SD, standard deviation.
***$p < 0.0001$ for difference between NHIS and VA.

RESULTS

The characteristics of the samples are given in Table 1. Relative to the NHIS sample, the VA sample had a higher mortality rate, and more individuals with medical comorbidity. This is consistent with what would be expected for the difference between an epidemiological sample (NHIS) and a clinic-based sample (VA). In addition, the VA sample had a shorter follow-up period with less variability, more men, more African Americans, and fewer Hispanics. Of note, the VA sample had a lower proportion of current drinkers, which would be expected for an older sample with increased medical comorbidity.

In both data sets, nondrinkers was the largest category. Most current drinkers reported drinking monthly or less, typically drinking 1 to 2 drinks per drinking day, and never binge drinking (Fig. 1). As the frequency of nonbinge drinking increased, a higher proportion of individuals reported drinking 3 to 4 drinks per drinking day and were categorized as binge drinkers.

We determined the adjusted hazard ratios (HRs) for all-cause mortality based on frequency of drinking for drinkers of 1 to 2 drinks per day in both the NHIS data and in the VA data. Frequency of low-level drinking is modeled as a continuous variable in the NHIS data set and as a discrete variable in the VA data set. Although our primary variable of interest was frequency of low-level drinking, all statistical models were also adjusted for typical drinking quantity and frequency of binge drinking. Fractional polynomial modeling was used in the NHIS data set to allow for flexible parameterization of nonlinear associations. Using a stepwise approach, frequency of low-level drinking in the NHIS resulted in a model containing terms for frequency and frequency cubed. Other polynomial terms, including $(\text{frequency})^2$, $\log(\text{frequency})$, $(\text{frequency})^{-1}$, and $(\text{frequency})^{-2}$, were not statistically significant. Estimated HR for alcohol-related variables are given in Table S2 and are plotted in Fig. 2.

In both data sets, the reference group was defined as the frequency of nonbinge drinking associated with the lowest risk of all-cause mortality: 3.2 times weekly for the NHIS sample and 2 to 3 times weekly for the VA sample. Despite the differences between the 2 data sets, the trends are the same. The minimum risk for all-cause mortality is estimated to be drinking 1 to 2 drinks approximately 2 to 3 times weekly, and risk of all-cause mortality increased as the number of drinking occasions increased. In the NHIS sample, using drinking 1 to 2 drinks 3.2 times weekly as the reference group, all-cause mortality is increased in a stepwise fashion to HR = 1.05 ($p = 0.003$) for drinking 1 to 2 drinks 5 times

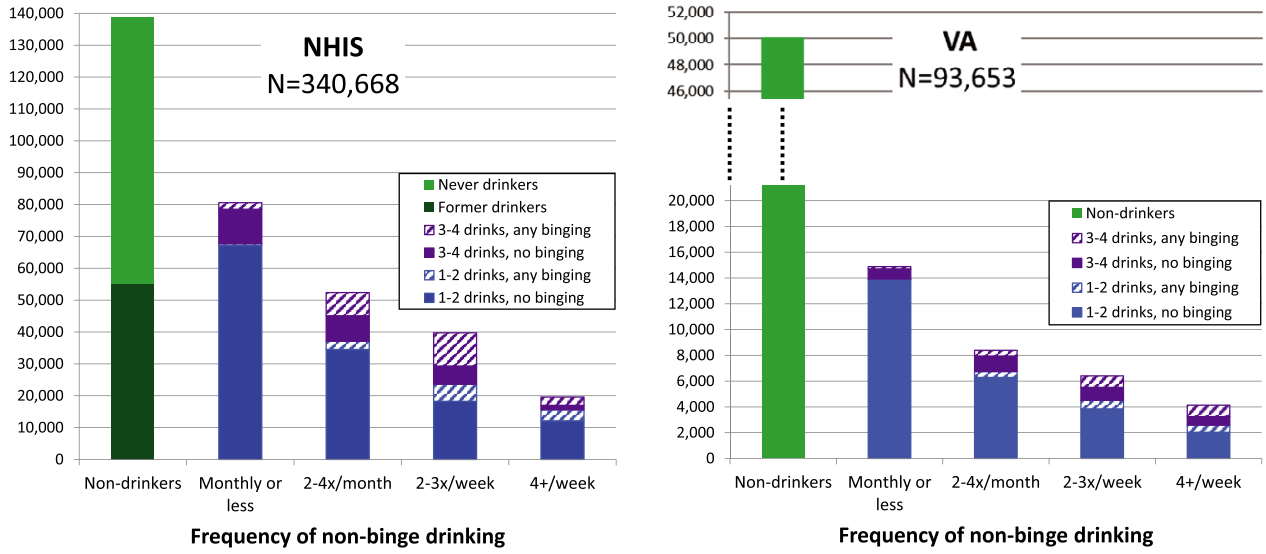

Fig. 1. Patterns of alcohol use for each sample.

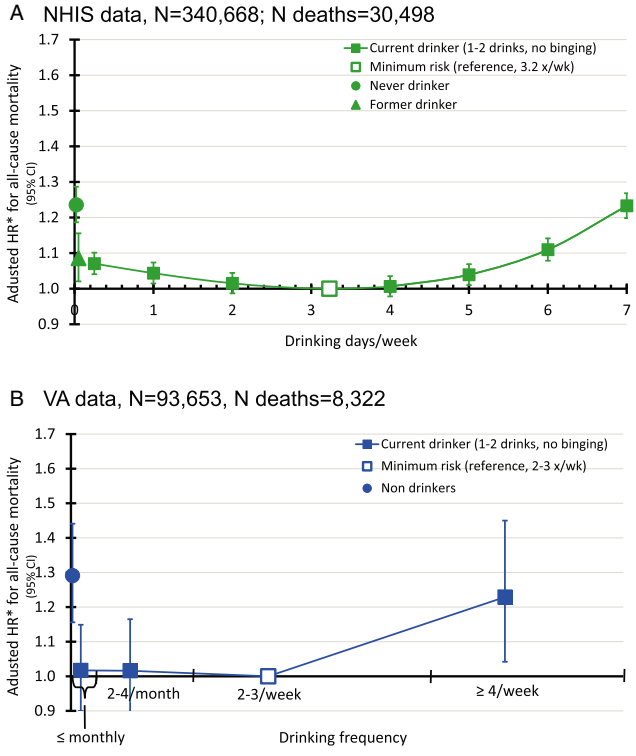

Fig. 2. Adjusted HR* of all-cause mortality by drinking frequency for individuals who drink 1 to 2 drinks on occasion and never binge drink. (**A**) NHIS data, *N* = 340,668; *N* deaths = 30,498. *NHIS HR are adjusted for birth cohort, gender, race, typical drinking quantity when not binge drinking, frequency of binge drinking, health and wellness factors (current smoking status, perceived health status, exercise level, and medical comorbidities), socioeconomic factors (educational attainment, employment status, and whether the household received food stamps in the last calendar year), region of country, and year of survey. (**B**) VA data, *N* = 93,653; *N* deaths = 8,322. *VA HR are adjusted for birth cohort, gender, race, typical drinking quantity when not binge drinking, frequency of binge drinking, and medical comorbidities.

weekly, HR = 1.12 ($p < 0.0001$) for drinking 1 to 2 drinks 6 times weekly, and HR = 1.26 ($p < 0.0001$) for drinking 1 to 2 drinks 7 times weekly. In the VA data, drinking 1 to 2 drinks 4 or more times weekly had an HR = 1.23 ($p = 0.01$) when compared to drinking 1 to 2 drinks 2 to 3 times weekly (the reference).

Relative to the reference, drinking less frequently also showed increased risk for all-cause mortality in the NHIS data, but not in the VA data. Specifically, in the NHIS data relative to drinking 3.2 times weekly, the increased risk in all-cause mortality was HR = 1.04 ($p = 0.05$) for individuals who drank once weekly and HR = 1.06 ($p < 0.01$) for individuals who drank once monthly. In the VA data, relative to drinking 2 to 3 times weekly, drinking 1 to 2 drinks 2 to 4 times monthly (HR = 1.02, $p = 0.8$) or less than monthly (HR = 1.02, $p = 0.8$) did not have a statistically significant increased risk of all-cause mortality.

Because unmeasured confounders may be different between current drinkers and nondrinkers, nondrinkers were evaluated separately and were found to have increased risk of mortality relative to current drinkers who drink 1 to 2 drinks per occasion and never binge. In the NHIS data, increased risk for all-cause mortality was seen in never drinkers (HR = 1.24, $p < 0.0001$) and in former drinkers (HR = 1.14, $p < 0.0001$) relative to drinking 1 to 2 drinks 3.2 times weekly. In the VA data set, increased risk for all-cause mortality was seen in nondrinkers (HR = 1.29, $p < 0.0001$), relative to drinking 1 to 2 drinks 2 to 3 times weekly. There was no variable in the VA data to distinguish between never drinkers and former drinkers.

Using the causes of mortality available in the NHIS data, we evaluated the association between the frequency of non-binge drinking with risk of cardiovascular mortality and risk of cancer-related mortality (Fig. 3). A J-shaped relationship was seen between frequency of nonbinge drinking and risk of

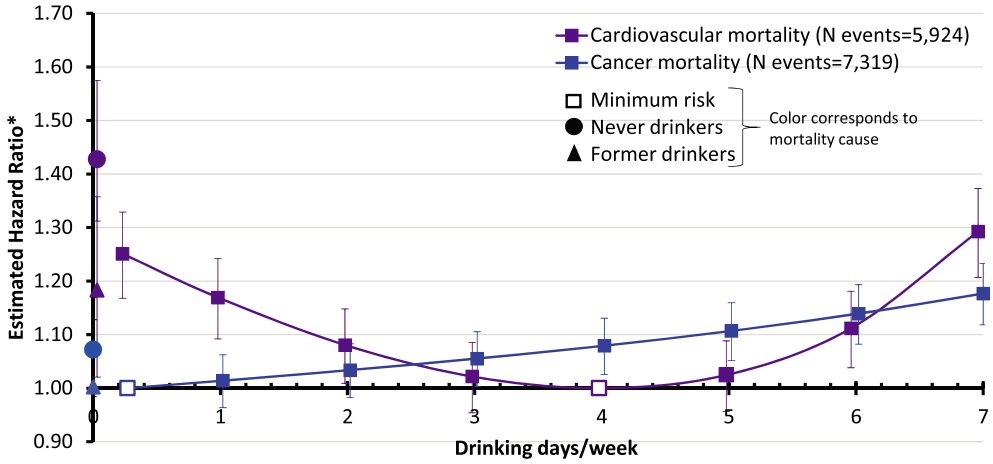

Fig. 3. Estimated HR for mortality due to cardiovascular disease and cancer based on the frequency of nonbinge drinking. Curves correspond to risk for individuals who typically drink 1 to 2 drinks per occasion and never binge in the NHIS data set. *HR are adjusted for birth cohort, gender, race, typical drinking quantity when not binge drinking, frequency of binge drinking, health and wellness factors (current smoking status, perceived health status, exercise level, and medical comorbidities), socioeconomic factors (educational attainment, employment status, and whether the household received food stamps in the last calendar year), region of country, and year of survey.

cardiovascular mortality: The minimum risk was seen in individuals who drank 1 to 2 drinks 4 times weekly. In contrast, the risk of cancer-related mortality increased linearly with frequency of nonbinge drinking (i.e., the minimum risk was in individuals who drank 1 to 2 drinks once monthly, and in former drinkers).

There is strong evidence that alcohol-related health effects differ between men and women (Klatsky and Udaltsova, 2007; Zheng et al., 2017); therefore, stratified analyses were run in the NHIS data (Fig. 4) and in the VA data. The results from the stratified analysis in the VA data are not shown because extremely wide confidence intervals in women suggest lack of power. Increased mortality risk was seen with daily or near-daily nonbinge drinking in both men and women, although men and women differed with respect to the nonbinge drinking frequency at which all-cause and cardiovascular mortality risks were minimized: For all-cause mortality, the minimum risk for women was at a lower frequency of drinking (1 to 2 drinks 2.7 times weekly) compared to men (1 to 2 drinks 3.4 times weekly). For cardiovascular mortality, the gender differences were reversed, with the minimum risk for women being 1 to 2 drinks 4.3 times weekly and for men 1 to 2 drinks 4.0 times weekly. With respect to cancer mortality, minimum risk was drinking 1 to 2 drinks once monthly for both women and men, the minimum frequency for current drinkers. In the VA data set, there were no statistically significant interactions between gender and drinking patterns in terms of all-cause mortality (the VA data set did not have causes of death to partition this by cardiovascular and cancer mortality).

Smoking behavior is confounded with alcohol use and increases the risk of mortality. Although all analyses were adjusted for smoking behavior in the NHIS sample, we also specifically evaluated the association between frequency of nonbinge drinking and mortality in the NHIS subsample of individuals who never smoked 100 cigarettes in their lifetime (N = 155,308). Consistent with the overall data set, among never smokers who typically drank 1 to 2 drinks at a time and never binged, increased all-cause mortality was seen with daily or near-daily drinking (Fig. S1) relative to the never smokers who drank the same amount, but only 3 to 4 d/wk.

DISCUSSION

Using 2 large, independent data sets, this study identified an increased risk in mortality among individuals who drink 1 to 2 drinks 4 or more times weekly compared to individuals who drink 1 to 2 drinks 3 times weekly, despite the fact that both groups may be drinking within the current U.S. dietary guidelines of up to 1 drink daily for women and up to 2 drinks daily for men (Division of Population Health, 2016; National Institute on Alcohol Abuse and Alcoholism, 2016; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Although other studies have evaluated the association between drinking frequency and mortality (Costanzo et al., 2010a,b; Foster and Marriott, 2006; Mukamal et al., 2003), the unique aspect of our study is in identifying the nadir of all-cause mortality associated with the frequency of low-level drinking and using that nadir as the reference category for comparison, rather than using nondrinkers as the reference category.

In both the NHIS data set, an epidemiologic sample, and VA data set, a clinical sample, the nadir of all-cause mortality is drinking 1 to 2 drinks approximately 3 times weekly, and drinking 1 to 2 drinks more frequently increases the risk of all-cause mortality. In our analyses of the NHIS and VA data, drinking daily does not show increased risk of all-cause mortality relative to nondrinkers, which is consistent with previously published studies, and is the reason for the J- or U-shaped curve often discussed in the literature (Costanzo

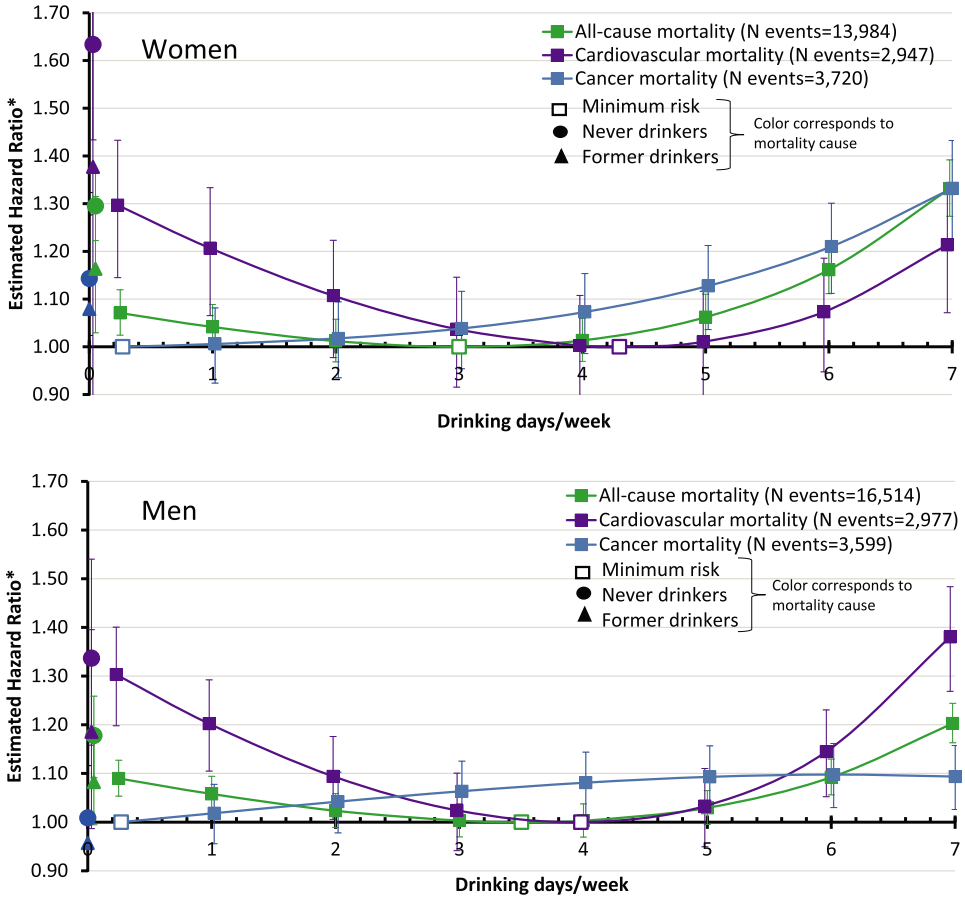

Fig. 4. Gender-stratified HR for mortality based on frequency of nonbinge drinking. Curves correspond to risk for individuals who typically drink 1 to 2 drinks per occasion and never binge in the NHIS data set. *HR are adjusted for birth cohort, gender, race, typical drinking quantity when not binge drinking, frequency of binge drinking, health and wellness factors (current smoking status, perceived health status, exercise level, and medical comorbidities), socioeconomic factors (educational attainment, employment status, and whether the household received food stamps in the last calendar year), region of country, and year of survey.

et al., 2010a,b). However, the reference category with the lowest risk level of alcohol consumption is essential for making recommendations regarding alcohol use. For example, the current U.S. dietary guidelines recommend that nondrinkers remain abstinent from alcohol (Division of Population Health, 2016; National Institute on Alcohol Abuse and Alcoholism, 2016; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015), and it is appropriate to guide current drinkers based on the minimum all-cause mortality seen based on drinking patterns among those who drink.

In addition to analyzing all-cause mortality, we examined cardiovascular and cancer mortality in the NHIS data set. Cardiovascular mortality is U-shaped with a nadir of drinking 3 to 4 d/wk; however, cancer-related mortality is minimized at the lowest level of alcohol consumption, and risk linearly increases with frequency of drinking, even at low levels of drinking. This differential effect of drinking on causes of mortality highlights an opportunity for precision prevention: Individuals with a strong family history of cardiovascular disease may reduce risk by drinking a few days per week whereas individuals with a strong family history of cancer may be cautioned not to drink at all to minimize risk.

A study was recently published by Xi and colleagues (2017) that analyzed the same NHIS data included here. Using the reference group of never drinkers, the study reported decreased risk of all-cause mortality with both light drinking (12 drinks yearly to less than 3 drinks weekly) and low-level drinking (3 to 14 drinks weekly for men and 3 to 7 drinks weekly for women). In addition to differing categories of alcohol use, Xi and colleagues (2017). used slightly different covariates (a detailed comparison of the 2 studies is given in the online Appendix). The current study extends their findings by examining the relative effect of frequency of alcohol use within a set of light and moderate drinkers.

Our analytic strategy sought to minimize the possibility that the observed effects of alcohol on mortality are due to confounding. All analyses were adjusted for age,

gender, race, comorbidity, drinking quantity, and binge drinking frequency. In addition, we adjusted for smoking status in the NHIS sample (smoking status was not available in the VA sample). Finally, we repeated the analyses in the NHIS subset of never smokers. Because the findings were robust across analyses, the observed associations are unlikely to be due to these potential confounders. However, other unmeasured confounders may have influenced the results. For example, psychosocial stressors that may disproportionately and adversely affect individuals of low socioeconomic status may negatively affect health outcomes. Therefore, the inability to adjust for these factors may bias the results. In addition, the VA sample had a higher proportion of individuals with comorbidities, and because some of them may have reduced drinking after development of certain illnesses, the inability to differentiate former drinkers from lifetime abstainers is important to note.

The data sets used in this study are large and have complementary designs, but there are potential limitations. First, both studies relied on in-person measurements of self-reported alcohol use, rather than anonymous reports. This is of concern because when comparing alcohol use from in-person surveys to anonymous surveys, the in-person surveys show under reporting of alcohol use (Del Boca and Darkes, 2003; Polich, 1982). In addition, although outcomes were measured at the end of the study period, alcohol use patterns were measured once. This likely resulted in higher variance than if repeated measures of use were available.

In summary, this report demonstrates an association between increased mortality and drinking behaviors that falls within the current U.S. dietary guidelines for "healthy" alcohol use. Consuming 1 to 2 drinks at a time on 5 or more occasions weekly was associated with elevated risk of all-cause mortality, relative to drinking less frequently. This finding was observed in both a large epidemiological sample and a large clinic population and suggests that the guidelines for "healthy" alcohol use should be lowered.

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CONFLICT OF INTEREST

Dr. Bierut is listed as an inventor on Issued U.S. Patent 8080371, "Markers for Addiction" covering the use of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction. The remaining authors do not have any conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Estimated HRs for all-cause mortality based on frequency of nonbinge drinking among never smokers, individuals who smoked fewer than 100 cigarettes lifetime. Curves correspond to risk for individual never-smokers who typically drink 1 to 2 drinks per occasion and never binge in the NHIS data set.

Table S1. Categorization of nonbinge drinking frequency in the VA data.

Table S2. Estimated HRs, *p*-values, and corresponding 95% confidence intervals (CIs) for all-cause mortality in the NHIS data (A) and in the VA data (B). In each analysis, the reference low-level drinking frequency was set to be the low-level drinking frequency with the minimum risk for all-cause mortality.