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Marine ω-3 Polyunsaturated Fatty Acid Intake and Risk of Colorectal Cancer Characterized by Tumor-Infiltrating T Cells

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IMPORTANCE Marine ω-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid, docosahexaenoic acid, and docosapentaenoic acid, possess potent immunomodulatory activity and may protect against cancer development. However, evidence relating marine ω-3 PUFAs to colorectal cancer (CRC) risk remains inconclusive.

OBJECTIVE To test the hypothesis that marine ω-3 PUFA intake may be associated with lower risk of CRC subsets characterized by immune infiltrate.

DESIGN, SETTING, AND PARTICIPANTS This prospective cohort study was conducted among participants in the Nurses' Health Study (1984-2010) and Health Professionals Follow-up Study (1986-2010).

EXPOSURES Intake of marine ω-3 PUFAs.

MAIN OUTCOMES AND MEASURES Incidence of CRC characterized by $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, or $FOXP3^+$ T-cell densities in tumor tissue, measured by immunohistochemical and computer-assisted image analysis.

RESULTS Among 173 229 predominantly white participants, 125 172 with 2 895 704 person-years of follow-up provided data about marine ω-3 PUFA intake every 4 years through a validated food frequency questionnaire and followed up for incident CRC evaluation. Of 2504 CRC cases, we documented 614 (252 men, 362 women) from which we could assess T-cell infiltration in the tumor microenvironment. The inverse association of marine ω-3 PUFAs intake with CRC risk differed according to $FOXP3^+$ T-cell infiltration: compared with intake of less than 0.15 g/d of marine ω-3 PUFAs, intake of at least 0.35 g/d was associated with a multivariable hazard ratio (HR) of 0.57 (95% CI, 0.40-0.81; $P < .001$ for trend) for $FOXP3^+$ T-cell-high tumors. In contrast, the HR for $FOXP3^+$ T-cell-low tumors was 1.14 (95% CI, 0.8-1.60) ($P = .77$ for trend; $P = .01$ for heterogeneity). No statistically significant differential association was found for high-density tumors (compared with low-density tumors) according to $CD3^+$, $CD8^+$, or $CD45RO^+$ cell density ($P \geq .34$ for heterogeneity for all comparisons). In functional assays, the suppressive activity of regulatory T cells was approximately 2-fold lower (T-effector-cell proliferation, ≥64% vs 38%) when preincubated with docosahexaenoic acid at 50μM, 100μM, and 200μM concentrations than without docosahexaenoic acid ($P < .001$ for all comparisons).

CONCLUSIONS AND RELEVANCE High marine ω-3 PUFA intake was associated with lower risk of CRC with high-level, but not low-level, $FOXP3^+$ T-cell density, suggesting a potential role of ω-3 PUFAs in cancer immunoprevention through modulation of regulatory T cells.

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Substantial experimental evidence demonstrates that ω-3 polyunsaturated fatty acids (ω-3 PUFAs), namely eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA), exert potent anti-inflammatory effects and protect against the development of colorectal cancer (CRC).[1] However, human data relating ω-3 PUFA intake to CRC risk remain inconclusive, with a null association reported in all[2-10] but one[11] prospective study. Recently, we found that the inverse association between intake of marine ω-3 PUFAs and CRC was confined to the 10% to 15% of tumors with high microsatellite instability (MSI)[12] caused by loss of DNA mismatch repair activity.[13] These data suggest that the anticancer effects of ω-3 PUFAs may vary by tumor subtypes and require further characterization. Understanding the mechanisms underlying such differential benefits may lead to more effective precision medicine–based chemopreventive strategies.

The multifaceted roles of marine ω-3 PUFAs in immune regulation have long been recognized, including suppression of T-cell proliferation and promotion of $CD4^+$ helper T cell, type 1 (T_H1) differentiation.[14] Likewise, growing evidence indicates that immune cells in the tumor microenvironment play an active role in cancer evolution.[15] A greater lymphocytic reaction to CRC has been associated with better prognosis,[16,17] whereas regulatory T (Treg) cells with prerequisite expression of the transcription factor *FOXP3* (OMIM: 300292) may support cancer development by reducing the host antitumor immune responses.[18-20] Moreover, the immune microenvironment of cancer is uniquely associated with tumor molecular features.[15] For example, MSI-high CRC is characterized by high infiltration of activated T_H1 cells and altered expression of immunomodulatory and immune checkpoint genes.[21-24]

Taken together, these data suggest (1) that marine ω-3 PUFAs may exert an antitumor effect through their immunomodulatory activity and (2) that our research group's previous finding of benefit of high marine ω-3 PUFA intake on MSI-high tumor development[12] may be owing to differences in tumor-infiltrating immune cells associated with MSI status. Hence, we hypothesized that the inverse association of marine ω-3 PUFA intake with CRC might be stronger for certain immune subtypes of tumors. To test this hypothesis, we classified CRC cases according to the density of T cells in the tumor microenvironment and investigated whether the association of prediagnostic marine ω-3 PUFA intake with CRC differed by these subtypes. We then corroborated our findings with experimental studies to examine the mechanisms by which marine ω-3 PUFAs influence T-cell function.

Key Points

Question Is marine ω-3 polyunsaturated fatty acid intake differentially associated with risk of colorectal cancer (CRC) subsets characterized by immune infiltrate?

Findings In this cohort study, high intake of marine ω-3 polyunsaturated fatty acids was associated with lower risk of CRC with high-level $FOXP3^+$ T-cell density. Marine ω-3 polyunsaturated fatty acids decreased the in vitro suppressive activity of colonic regulatory T cells.

Meaning These findings suggest a potential role of marine ω-3 polyunsaturated fatty acids in the immunoprevention of CRC through modulation of regulatory T cells.

Methods

Study Population

Details about the cohorts of the Nurses' Health Study (NHS) (started in 1976, and including 121 700 women aged 30-55 years) and Health Professionals Follow-up Study (HPFS) (initiated in 1986, and including 51 529 men aged 40-75 years) have been described elsewhere.[25,26] Briefly, participants completed a detailed questionnaire about their medical history and lifestyle at baseline and every 2 years thereafter. The response rates through 2010 were 95.4% in the NHS and 95.9% in the HPFS for each of the questionnaires. Dietary data were collected and updated using the food frequency questionnaires (FFQs) every 4 years. In the present analysis, we used year 1984 for the NHS and year 1986 for the HPFS as baseline, when we first collected detailed data on ω-3 PUFA intake. Among 81 761 women and 49 938 men who returned the baseline FFQ, we excluded 4454 women and 1997 men who had a history of cancer at baseline and 59 women and 17 women who left more than 70 blank responses on the baseline FFQ, had missing information about marine ω-3 PUFA intake, or reported implausible energy intake (<600 or >3500 kcal/d for women, <800 or >4200 kcal/d for men). After exclusions, 77 248 women and 47 924 men were eligible for analysis. We obtained written informed consent from all participants. This study was approved by the institutional review board at Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health.

Assessment of Marine ω-3 PUFA Intake

Detailed description of ω-3 PUFA assessment is provided in the Supplement.[10,27] In each FFQ, we asked participants how often, on average, they consumed each food of a standard portion size during the previous year. We calculated the average daily intake for each nutrient by multiplying the reported frequency of consumption of each item by its nutrient content and then summing across from all foods. We adjusted nutrient intake for total caloric intake using the nutrient residual method.[28] Marine ω-3 PUFA intake was calculated by summing EPA, DHA, and DPA consumption. Use of fish oil supplement was also assessed and included in the estimation of marine ω-3 PUFA intake. To estimate long-term habitual consumption, we calculated the cumulative average of marine ω-3 PUFA intake during follow-up using all of these dietary measures. The FFQs have demonstrated good reproducibility and validity in assessing marine ω-3 PUFA intake,[29,30] as described in the Supplement.

Ascertainment of CRC Cases

In both cohorts, cancer diagnoses were reported by participants on the biennial questionnaires. Deaths were reported by

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family members or the postal system, or they were identified through a search of the National Death Index. With consent from participants or next of kin, medical records were obtained and reviewed by study physicians, who were blinded to exposure information, to confirm CRC diagnosis and extract information on anatomic location, stage, and histologic type. We retrieved formalin-fixed paraffin-embedded tissue blocks for immunity assessment from hospitals throughout the United States where participants had undergone surgical resection. Through 2010, we documented 1488 CRC cases in the NHS and 1016 cases in the HPFS. Tissue specimens were successfully collected and assayed for T-cell density measurements from 362 patients in the NHS and 252 patients in the HPFS.

Tumor Tissue Analyses

Tissue microarrays were constructed to assess the density of $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, and $FOXP3^+$ T cells in tumor tissue, as previously described.[31-33] We used immunohistochemical analysis and an automated scanning microscope and Ariol image analysis system (Genetix Corp) to calculate the average density (cells/mm^2) of each T-cell marker in tissue microarray cores. We dichotomized cases based on the median density of each T-cell marker among all cases. Methods of MSI assessment[34] are provided in the Supplement.

In Vitro T-Cell Suppression Assay

Colonic lamina propria $FOXP3^+$ Treg cells were isolated from C57BL/6J wild-type mice bred and housed in microisolator cages in a barrier facility as previously described[35] and preincubated for 4 hours with different concentrations of DHA (*cis*-4,7,10,13,16,19-docosahexaenoic acid) (Sigma-Aldrich) in complete RPMI 1640 medium (Sigma-Aldrich) containing 0.5% bovine serum albumin. The DHA was removed by washing cells with RPMI medium containing 1% fetal bovine serum. We used CellTrace molecular probes (CellTrace Violet Cell Proliferation Kit; ThermoFisher Scientific) to monitor distinct generation of proliferating cells by dye dilution in flow cytometry. Isolated $CD4^+IL2RA^-$ T effector (Teff) cells were incubated with CellTrace (5 µM/mL) at 37°C for 20 minutes and washed with prewarmed RPMI 1640 containing 5% fetal bovine serum. Then, DHA-treated Treg cells were cultured with CellTrace-stained $CD4^+IL2RA^-$ Teff cells (Treg to Teff ratio, 1:4) in the presence of anti-CD3 antibody (5 µg/mL) and anti-CD28 antibody (BioLegend) (5 µg/mL) in 96-well round plates for 48 hours at 37°C. The proliferation percentage of Teff cells was analyzed by flow cytometry.[36] More details about flow cytometry and cell sorting are provided in the Supplement. Experiments using mice were approved by and carried out in accordance with Harvard Medical School's Standing Committee on Animals and the National Institutes of Health guidelines for animal use.

Statistical Analysis

Our primary hypothesis testing was the heterogeneity test between the association of marine ω-3 PUFA intake with lymphocyte-rich CRC compared with the association of marine ω-3 PUFA intake with lymphocyte-poor CRC. All other assessments, including evaluation of individual hazard ratio (HR) estimates represent secondary analyses. More details about statistical analysis are provided in the Supplement.

Participants were followed up from the age at which the baseline questionnaire was returned until the age at the date of death, CRC diagnosis, loss to follow-up, or end of follow-up (June 1, 2010, for the NHS; January 31, 2010, for the HPFS), whichever came first. For overall CRC risk, we used a time-varying Cox proportional hazards regression model to estimate HR and 95% CIs associated with marine ω-3 PUFA intake. We first performed the analyses in each cohort separately. Because no appreciable difference was detected by cohort (comparing ≥0.35 g/d with <0.15 g/d: HR, 1.02 [95% CI, 0.85-1.23] in the HPFS cohort vs HR, 1.07 [95% CI, 0.91-1.26] in the NHS cohort [$P = .13$ for heterogeneity]), we then conducted a pooled analysis in the combined cohort.

For subtype-specific CRC risk, we fitted a cause-specific Cox proportional hazards regression model with a duplication method for competing risk data to compute HR and 95% CIs.[37,38] A heterogeneity test was performed using a likelihood ratio test by comparing the model in which the association with marine ω-3 PUFAs was allowed to vary by tumor subtypes with a model in which all the associations were held constant. We stratified all analyses by age, sex, and year of questionnaire return. We conducted the multivariable analysis by adjusting for several risk factors of CRC. Details about covariate assessment are provided in the Supplement.

Results

Characteristics of Study Participants

Among 125 172 participants with 2 895 704 person-years of follow-up, we documented 2504 CRC cases, of which 614 had available tissue and data of T-cell density in tumor tissue. As summarized in **Table 1**, participants with higher marine ω-3 PUFA intake were less likely to be current smokers and were more likely to engage in physical activity, undergo endoscopy, use multivitamins, and consume a healthy diet.

Association of Marine ω-3 PUFAs With CRC Characterized by Tumor-Infiltrating T-Cell Density

Consistent with our group's previous report,[10] we found that marine ω-3 PUFA intake was not statistically significantly associated with CRC risk, after adjusting for other risk factors, comparing participants with at least 0.35-g/d intake with those with less than 0.15-g/d intake (HR, 0.85 [95% CI, 0.67-1.09]; $P = .07$ for trend; **Table 2**). Similar results were observed among cases without tumor immunity data (eTable 1 in the Supplement). We then classified CRC cases into subtypes with low-level vs high-level infiltrate of each of the 4 T-cell markers and tested our primary hypothesis that the association between marine ω-3 PUFA intake and CRC risk differed according to T-cell densities in tumor tissues. To account for multiple-hypothesis testing, we adjusted the statistical significance level to $\alpha = .012$ (approximately .05/4). We found that the beneficial association of high marine ω-3 PUFA intake appeared confined to cancers with high infiltration of $FOXP3^+$ T cells:

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Table 1. Age-Standardized Participant Characteristics According to Intake of Marine ω-3 PUFAs[a]

Characteristic	Marine ω-3 PUFA Intake, g/d: Women <0.15	Women 0.15-0.24	Women 0.25-0.34	Women ≥0.35	Men <0.15	Men 0.15-0.24	Men 0.25-0.34	Men ≥0.35
Participants, No.	35 176	17 461	12 895	11 716	14 691	10 444	9288	13 501
Intake of marine ω-3 PUFAs, g/d	0.08	0.19	0.29	0.62	0.08	0.20	0.29	0.68
Mean age, y	62.1	60.3	60.7	62.2	61.8	61.8	62.2	63.8
Mean BMI	26.2	26.1	26.0	26.2	25.8	25.7	25.7	25.6
Mean physical activity, MET-hours/wk[b]	15.1	17.7	19.0	20.9	31.7	33.4	35.0	37.3
Mean pack-years of smoking before age 30 years	6.9	6.9	7.0	7.1	11.3	11.0	10.8	10.9
Current smoker, %	14	13	12	11	7	6	5	4
Colorectal cancer in a parent or sibling, %	17	18	17	17	14	14	14	14
History of endoscopy, %	25	26	28	30	23	25	27	29
Current multivitamin use, %	52	54	55	60	48	49	51	56
Regular aspirin or NSAID use, %[c]	52	52	51	53	50	50	51	52
Postmenopausal, %	81	81	82	82	NA	NA	NA	NA
Current hormone use, %[d]	51	52	51	46	NA	NA	NA	NA
Mean dietary intake								
ω-3 PUFAs, g/d	1.08	1.21	1.32	1.72	1.22	1.35	1.46	1.92
18:3 ω-3 (ALA), g/d	0.96	0.97	0.98	1.07	1.13	1.13	1.14	1.20
20:5 ω-3 (EPA), mg/d	21	58	92	235	21	59	94	257
22:5 ω-3 (DPA), mg/d	12	18	23	33	13	20	25	40
22:6 ω-3 (DHA), mg/d	49	117	175	349	49	118	174	381
ω-6 PUFAs, g/d	9.03	8.94	8.83	9.07	12.2	12.0	11.8	11.8
Fish, servings/wk	0.8	1.8	2.4	3.7	0.7	1.6	2.1	3.9
Total red meat, servings/wk	5.4	5.3	4.8	4.0	7.1	7.1	6.2	4.9
Alcohol, g/d	4.8	6.3	6.6	6.1	10.4	12.4	12.5	11.3
Folate, μg/d	526	532	558	631	580	588	616	713
Calcium, mg/d	1157	1163	1202	1303	983	968	973	1039
Vitamin D, IU/d	378	408	457	566	370	404	444	580
Total fiber, g/d	18.1	18.8	19.5	20.4	21.7	22.2	23.0	24.7
AHEI score	44.6	46.5	48.5	51.6	38.7	41.5	43.1	46.8

Abbreviations: AHEI, Alternative Healthy Eating Index; ALA, α-linolenic acid; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MET, metabolic equivalent; NA, not applicable; NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid.

[a] Updated information throughout follow-up was used to calculate the mean for continuous variables and percentage for categorical variables. All variables are age standardized except age.

[b] Physical activity is represented by the product sum of the MET of each specific recreational activity and hours spent on that activity per week.

[c] Regular users are defined as those who take 2 or more standard 325-mg tablets of aspirin or 2 or more tablets of NSAIDs per week.

[d] Proportion of current menopausal hormone use is calculated among postmenopausal women only.

compared with intake of less than 0.15 g/d of marine ω-3 PUFAs, intake of at least 0.35 g/d was associated with a multivariable HR of 0.57 (95% CI, 0.40-0.81) (*P* < .001 for trend) for FOXP3$^+$ T-cell-high tumors. In contrast, the corresponding HR was 1.14 (95% CI, 0.81-1.60) (*P* = .77 for trend) for FOXP3$^+$ T-cell-low tumors (*P* = .01 for heterogeneity). Similar heterogeneity was observed for each individual PUFA (EPA, DHA, and DPA; eTable 2 in the Supplement). We did not observe statistically significant heterogeneity for high-density vs low-density tumors classified by CD3$^+$, CD8$^+$, or CD45RO$^+$, and we obtained similar results from analyses conducted within each cohort separately (eTables 3 and 4 in the Supplement). We assessed the dose-response relationship using spline analysis and found that marine ω-3 PUFA intake appeared linearly associated with lower risk of FOXP3$^+$ T-cell-high cancer (HR per 0.15-g/d increment, 0.76 [95% CI, 0.65-0.90]; *P* = .001), but not with FOXP3$^+$ T-cell-low cancer (HR per 0.15-g/d increment, 0.96 [95% CI, 0.82-1.12]; *P* = .58) (eFigure 1 in the Supplement).

In secondary analyses, given our previous finding that marine ω-3 PUFA intake was inversely associated with MSI-high CRC but not microsatellite-stable (MSS) cancer,[12] we further classified tumors into 4 subtypes jointly according to FOXP3$^+$ T-cell infiltration and MSI status. As detailed in **Table 3**, high marine ω-3 PUFA intake appeared to be associated with lower risk of FOXP3$^+$ T-cell-high tumors regardless of MSI status, although the number of MSI-high cases was limited. By comparing T-cell density between MSS and MSI-high tumors, we found that CD45RO$^+$ and FOXP3$^+$ T cells were more enriched in MSI-high than MSS tumors[31] (eTable 5 in the Supplement).

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Table 2. Risk of CRC, Overall and by Tumor-Infiltrating T-Cell Subset Density, According to Intake of Marine ω-3 PUFAs Among Study Participants

Characteristic	Intake of Marine ω-3 PUFAs, g/d				*P* for Trend[a]	*P* for Heterogeneity[b]
	<0.15	0.15-0.24	0.25-0.34	≥0.35		
Overall[c]						
Person-years	931 900	798 692	539 046	629 860	NA	NA
Cases, No. (n = 614)	205	188	93	128	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.02 (0.84-1.24)	0.74 (0.58-0.95)	0.81 (0.64-1.01)	.02	NA
Multivariable HR (95% CI)[e]	1 [Referent]	1.00 (0.82-1.23)	0.74 (0.57-0.95)	0.85 (0.67-1.09)	.07	NA
CD3⁺ Cells						
Low density						
Cases, No. (n = 301)	104	92	49	56	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.99 (0.75-1.32)	0.79 (0.56-1.11)	0.70 (0.50-0.98)	.02	.34
Multivariable HR (95% CI)[e]	1 [Referent]	0.98 (0.73-1.30)	0.78 (0.55-1.10)	0.73 (0.52-1.03)	.05	.34
High density						
Cases, No. (n = 302)	100	88	43	71	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.97 (0.73-1.30)	0.69 (0.48-0.99)	0.91 (0.66-1.25)	.31	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.95 (0.71-1.28)	0.68 (0.47-0.98)	0.95 (0.69-1.32)	.49	NA
CD8⁺ Cells						
Low density						
Cases, No. (n = 290)	99	92	40	59	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.02 (0.76-1.35)	0.69 (0.47-0.99)	0.82 (0.59-1.15)	.10	.89
Multivariable HR (95% CI)[e]	1 [Referent]	0.99 (0.75-1.32)	0.67 (0.46-0.97)	0.86 (0.61-1.20)	.17	.90
High density						
Cases, No. (n = 302)	101	90	50	61	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.00 (0.75-1.33)	0.80 (0.57-1.12)	0.75 (0.54-1.04)	.05	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.98 (0.73-1.30)	0.79 (0.56-1.11)	0.78 (0.56-1.09)	.11	NA
CD45RO⁺ Cells						
Low density						
Cases, No. (n = 310)	92	107	42	69	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.27 (0.96-1.69)	0.72 (0.50-1.04)	0.88 (0.64-1.22)	.13	.72
Multivariable HR (95% CI)[e]	1 [Referent]	1.26 (0.95-1.67)	0.71 (0.49-1.03)	0.94 (0.67-1.30)	.26	.73
High density						
Cases, No. (n = 304)	113	81	51	59	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.81 (0.61-1.08)	0.77 (0.55-1.07)	0.75 (0.54-1.03)	.06	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.80 (0.60-1.06)	0.77 (0.55-1.08)	0.79 (0.57-1.10)	.13	NA
FOXP3⁺ Cells						
Low density						
Cases, No. (n = 285)	82	92	42	69	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.25 (0.93-1.69)	0.85 (0.58-1.23)	1.11 (0.80-1.54)	.95	.005
Multivariable HR (95% CI)[e]	1 [Referent]	1.21 (0.90-1.64)	0.82 (0.56-1.20)	1.14 (0.81-1.60)	.77	.006
High density						
Cases, No. (n = 299)	122	80	46	51	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.72 (0.55-0.96)	0.62 (0.44-0.87)	0.55 (0.39-0.77)	<.001	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.71 (0.53-0.94)	0.61 (0.43-0.87)	0.57 (0.40-0.81)	<.001	NA

Abbreviations: CRC, colorectal cancer; HR, hazard ratio; MET, metabolic equivalents; PUFA, polyunsaturated fatty acid.

[a] Trend test was performed using the median intake of each category.

[b] Likelihood ratio test was used to compare the model that allows for separate associations for tumors with different subtypes to the model that assumes a common association across subtypes.

[c] Cases without tumor-infiltrating T-cell data were excluded. A few cases with missing data on specific T-cell subsets were excluded for analyses of individual T cells: 11 for CD3⁺ cells; 22 for CD8⁺ cells; and 30 for FOXP3⁺ cells.

[d] Cox proportional hazards model was used with stratification by age, sex (ie, cohort), and calendar year of current questionnaire cycle.

[e] Additionally adjusted for family history of colorectal cancer, history of endoscopy, pack-years of smoking before age 30 years (in women: 0, 0 to <5, and ≥5; in men: 0, >0 to <10, and ≥10), current smoking status, body mass index (continuous), physical activity (in women: 0 to <5, 5 to <11.5, 11.5 to <22, and ≥22 METs/wk; in men: 0 to <10, 10 to <22.5, 22.5 to <41.5, and ≥41.5 METs/wk), multivitamin use, regular use of aspirin or nonsteroidal anti-inflammatory drugs (≥2 tablets/wk), alcohol consumption (in women: 0 to <0.15, 0.15 to <2.0, 2.0 to <7.5, and ≥7.5 g/d; in men: 0 to <5, 5 to <10, 10 to <15, 15 to <30, and ≥30 g/d), calcium intake (in quartiles), and Alternative Healthy Eating Index (in quartiles).

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Table 3. Risk of CRC, Jointly Classified by Microsatellite Instability and Tumor-Infiltrating FOXP3+ T-Cell Density, According to Intake of Marine ω-3 PUFAs Among Study Participants[a]

FOXP3+ T Cells	Intake of Marine ω-3 PUFAs, g/d <0.15	0.15-0.24	0.25-0.34	≥0.35	*P* for Trend[b]	*P* for Heterogeneity[c]
Microsatellite Stable						
Low density						
Cases, No. (n = 251)[d]	70	85	37	59	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	1.36 (0.99-1.87)	0.87 (0.58-1.30)	1.10 (0.77-1.58)	.95	.01
Multivariable HR (95% CI)[e]	1 [Referent]	1.32 (0.96-1.82)	0.85 (0.57-1.27)	1.14 (0.79-1.64)	.89	.02
High density						
Cases, No. (n = 230)[d]	91	64	34	41	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.77 (0.56-1.07)	0.59 (0.39-0.87)	0.56 (0.38-0.82)	<.001	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.76 (0.55-1.05)	0.58 (0.39-0.87)	0.58 (0.39-0.86)	.003	NA
Microsatellite Instability High						
Low density						
Cases, No. (n = 30)[d]	12	6	4	8	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.52 (0.19-1.38)	0.52 (0.17-1.64)	0.89 (0.35-2.25)	.86	.34
Multivariable HR (95% CI)[e]	1 [Referent]	0.49 (0.18-1.32)	0.49 (0.16-1.55)	0.92 (0.36-2.36)	.94	.35
High density						
Cases, No. (n = 64)[d]	30	13	12	9	NA	NA
Age-adjusted HR (95% CI)[d]	1 [Referent]	0.49 (0.25-0.93)	0.74 (0.38-1.45)	0.47 (0.22-1.00)	.07	NA
Multivariable HR (95% CI)[e]	1 [Referent]	0.48 (0.25-0.91)	0.74 (0.37-1.45)	0.50 (0.23-1.07)	.10	NA

Abbreviations: CRC, colorectal cancer; HR, hazard ratio; MET, metabolic equivalent.

[a] Cases without data on either microsatellite instability or tumor-infiltrating FOXP3+ T-cell density were excluded.

[b] Trend test was performed using the median intake of each category.

[c] Likelihood ratio test with 1 degree of freedom was used to compare the model that allows for separate associations for tumors with the same microsatellite instability status but infiltrated with different densities of FOXP3+ T cells to the model that assumes a common association.

[d] Cox proportional hazards model was used with stratification by age, sex (ie, cohort), and calendar year of current questionnaire cycle.

[e] Additionally adjusted for family history of CRC, history of endoscopy, pack-years of smoking before age 30 years (in women: 0, 0 to <5, and ≥5; in men: 0, >0 to <10, and ≥10), current smoking status, body mass index (continuous), physical activity (in women: 0 to <5, 5 to <11.5, 11.5 to <22, and ≥22 METs/wk; in men: 0 to <10, 10 to <22.5, 22.5 to <41.5, and ≥41.5 METs/wk), multivitamin use, regular use of aspirin or nonsteroidal anti-inflammatory drugs (≥2 tablets/wk), alcohol consumption (in women: 0 to <0.15, 0.15 to <2.0, 2.0 to <7.5, and ≥7.5 g/d; in men: 0 to <5, 5 to <10, 10 to <15, 15 to <30, and ≥30 g/d), calcium intake (in quartiles), and Alternative Healthy Eating Index (in quartiles).

Marine ω-3 PUFAs Decrease the T-Cell Suppressive Activity of Treg Cells

Finally, to investigate the effects of marine ω-3 PUFAs on the T-cell suppressive activity of FOXP3+ Treg cells, we assessed T-cell proliferation by using colonic Treg cells preincubated with different concentrations of DHA. We observed that DHA decreased the suppressive capacity of colonic Treg cells on naïve CD4+ Teff-cell proliferation in a dose-dependent manner (**Figure**). The suppressive activity of Treg cells preincubated with DHA at 50μM, 100μM, and 200 μM concentrations was approximately 2-fold lower than Treg cells preincubated without DHA (Teff-cell proliferation, ≥64% vs 38%; $P < .001$).

Discussion

In 2 large prospective cohorts, we found that high intake of marine ω-3 PUFAs was associated with lower risk of FOXP3+ T-cell-high CRC, but it was not associated with FOXP3+ T-cell-low CRC. This differential association appeared to be independent of MSI status. Consistent with these epidemiologic findings, our in vitro experiment showed that marine ω-3 PUFA DHA reduced the T-cell suppressive activity of colonic Treg cells. Taken together, these data support an antitumor effect of marine ω-3 PUFAs on CRC that is, at least in part, mediated through immune modulation of Treg cells.

Despite compelling experimental evidence supporting an anti-inflammatory and antineoplastic effect of marine ω-3 PUFAs, human studies have generally not shown a strong association between high intake of marine ω-3 PUFAs and lower CRC risk.[2-10] If marine ω-3 PUFA intake indeed lowers CRC risk, identifying a specific tumor subtype that is prevented by marine ω-3 PUFAs can yield more precise risk reduction and may lead to an effective prevention strategy against such a tumor subtype.[39] Recent studies suggest that marine ω-3 PUFAs are predominantly associated with risk of proximal colon cancer.[8,10] Our recent molecular pathological epidemiology study[39] showed that high marine ω-3 PUFA intake was primarily associated with lower risk of MSI-high tumors, which predominantly arise from the proximal colon, and thus provided a potential explanation for the previously reported subsite heterogeneity.[12] However, a mechanistic explanation for this differential association with MSI-high CRC was unclear. Therefore, our current results substantially extend previous findings and provide novel evidence by demonstrating that

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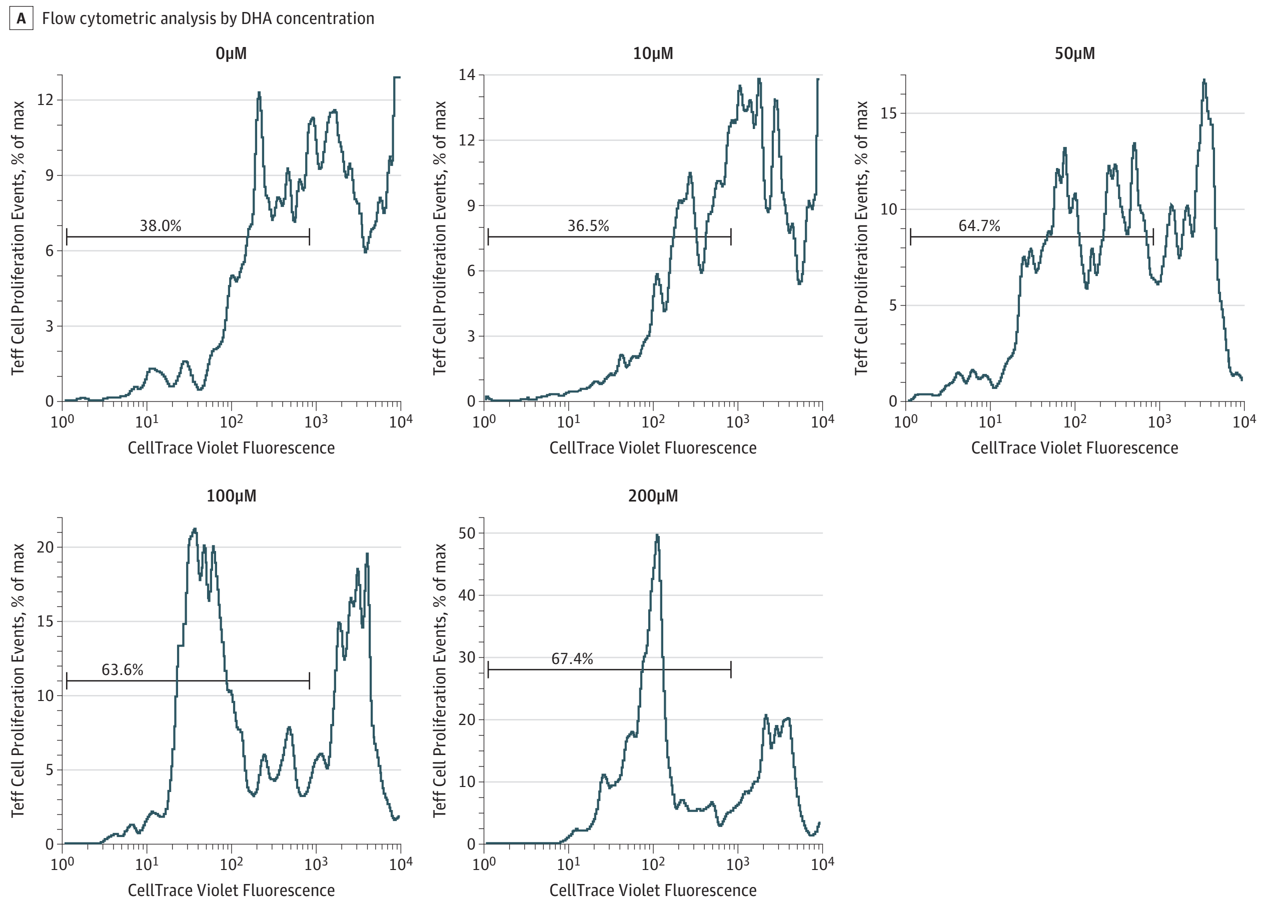
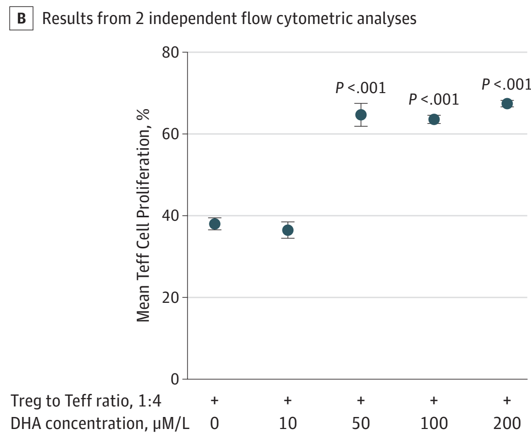
Figure. Docosahexaenoic Acid (DHA) Reduction of Suppressive Activity of Colonic Regulatory T (Treg) Cells

A Flow cytometric analysis by DHA concentration



B Results from 2 independent flow cytometric analyses



Before the analysis, colonic Treg cells were isolated and preincubated for 4 hours with different concentrations of DHA. Then, CellTrace-stained (CellTrace Violet Cell Proliferation Kit; ThermoFisher Scientific) $CD4^{+}IL2RA^{-}$ T effector (Teff) cells were coincubated for 48 hours with the DHA-treated Treg cells. The Teff cells then underwent flow cytometric analyses. A, Representative flow cytometric analyses of Teff cell proliferation events. The percentage-labeled horizontal lines represent gates, which show the proliferated cell populations; curves above 10^3 CellTrace fluorescence represent parent population rather than proliferated population. B, Illustration of mean (SEM) Teff cell proliferation from 2 independent analyses. *P* values are for unpaired, 2-tailed *t* tests compared with Teff coincubation with DHA-untreated (0 mg/L) Treg cells; plus signs indicate Treg + Teff combined in 1:4 ratio; max, maximum.

immune modulation may be a potential mechanism linking marine ω-3 PUFAs to lower CRC risk.

The colon is constantly exposed to a variety of environmental antigens from foods, resident microbiota, and pathogens, underscoring the critical importance of the intestinal immune system in maintaining a delicate balance between immunity and tolerance.[40] Perturbations of this immune homeostasis can result in chronic inflammation that can in turn

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increase cancer risk.[40] Gut Treg cells tune Teff immune responses, modulate the basal inflammatory tone of the intestine, and reset appropriate immune responses for the critical maintenance of intestinal homeostasis.[41] Although FOXP3$^+$ Treg cells can dampen aberrant immune responses against the resident gut microbiota or harmless dietary antigens, they may also suppress antitumor immune surveillance and facilitate cancer evasion.[19,42] Indeed, a high ratio of FOXP3$^+$ Treg cells and CD3$^+$ T cells has been shown to predict CRC risk.[43] The number of FOXP3$^+$ Treg cells is higher in human CRC than in surrounding unaffected mucosa,[44-46] and an enhanced infiltration of FOXP3$^+$ Treg cells has been detected in MSI-high CRCs compared with MSS cancers.[31,47] Moreover, in mouse models, transient ablation of FOXP3$^+$ Treg cells has been shown to decrease colorectal tumor burden through expansion of CD8$^+$ T cells.[18] Consistent with these data, a low density of FOXP3$^+$ T cells with a high density of CD8$^+$ T cells in CRC tissue has been associated with a favorable outcome in patients with CRC,[48,49] although the independent effect of FOXP3$^+$ Treg cells on CRC prognosis remains inconclusive.[17]

Marine ω-3 PUFAs exert immunomodulatory effects on T-cell proliferation and apoptosis.[50] In this study, we found that DHA decreased the suppressive functions of colonic Treg cells on Teff cell proliferation. These results are consistent with previous reports that a DHA-enriched diet curtails the suppressive activity of murine splenic Treg cells.[36] Collectively, these data support the hypothesis that marine ω-3 PUFAs protect against CRC through inhibition of the T cell–suppressive activity of Treg cells and therefore suggest the possibility of a strategy in which marine ω-3 PUFAs are used to blunt immunosuppressive Treg cells for the purpose of CRC prevention.[51]

Our study has several strengths, including the prospective design, repeated dietary assessment, long-term follow-up, and collection of detailed data on a variety of lifestyle risk factors for confounding control. Uniquely, our characterization of immune cells in tumor tissue enabled us to identify the specific cancer subtype that can be potentially prevented by intervention. Finally, our in vitro experiments provide a mechanistic correlate for our human data to support a causal basis for our observations.

The limitations of our study should also be noted. First, dietary assessment using FFQs is subject to measurement error. However, given the prospective design, any measurement error in ω-3 PUFA intake would have likely biased the observed associations toward the null and would be unlikely to explain the differential associations observed between subtypes of CRC. Second, despite our best efforts, we did not obtain tumor specimens from many patients for immunity evaluation. This is because we began retrieving tumor specimens in 1997 in the HPFS and in 2001 in the NHS. For many cases diagnosed earlier in follow-up, sufficient archived tumor tissue was not available for analysis. However, for this to lead to any bias in our main results, tissue availability would need to be associated with both cancer subtype and marine ω-3 PUFA intake–related CRC risk, which is unlikely. Indeed, as detailed in eTable 1 in the Supplement, we observed similar associations between marine ω-3 PUFA intake and CRC risk regardless of tissue availability, suggesting that lack of tissue data in some participants is unlikely to cause substantial bias. Third, we are aware of multiple-hypothesis testing, which is inherent in tumor subgroup analyses. Hence, we set subtype heterogeneity assessment (rather than evaluation of each subtype stratum) as our primary hypothesis testing and adjusted statistical significance level by Bonferroni correction. Finally, due to sparse data, some of the HRs were estimated with limited precision and should be interpreted cautiously.

Conclusions

In summary, we have shown that marine ω-3 PUFA intake is associated with lower risk of CRC that arises in the immune microenvironment with high, but not low, infiltration of FOXP3$^+$ T cells. High DHA reduces the T cell–suppressive activity of Treg cells in the in vitro model. Our data provide evidence that the anticancer effect of marine ω-3 PUFAs may be mediated through enhancing the immune response against cancer via modulation of FOXP3$^+$ Treg cells. Further studies are needed to determine the potential utility of marine ω-3 PUFAs as an immunomodulatory agent for CRC prevention.

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