REPORT

Long-term marine ω-3 polyunsaturated fatty acids intake in relation to incidence of colorectal cancer subclassified by macrophage infiltrates

Tomotaka Ugai,[1,2,17,*] Juha P. Väyrynen,[2,3,4,17] Satoko Ugai,[1,2,17] Rong Zhong,[1,2,5,17] Koichiro Haruki,[2] Mai Chan Lau,[2] Melissa Zhao,[2] Yuxue Zhong,[2] Qian Yao,[2] Kosuke Matsuda,[2] Jennifer L. Guerriero,[6] Kai Wang,[1] Xuehong Zhang,[7,8] Andrew T. Chan,[7,9,10,11] Charles S. Fuchs,[12] Marios Giannakis,[3,13,14] Edward L. Giovannucci,[1,8] Jeffrey A. Meyerhardt,[3] Mingyang Song,[1,9,10,18] Jonathan A. Nowak,[2,18] and Shuji Ogino[1,2,13,15,16,18,*]

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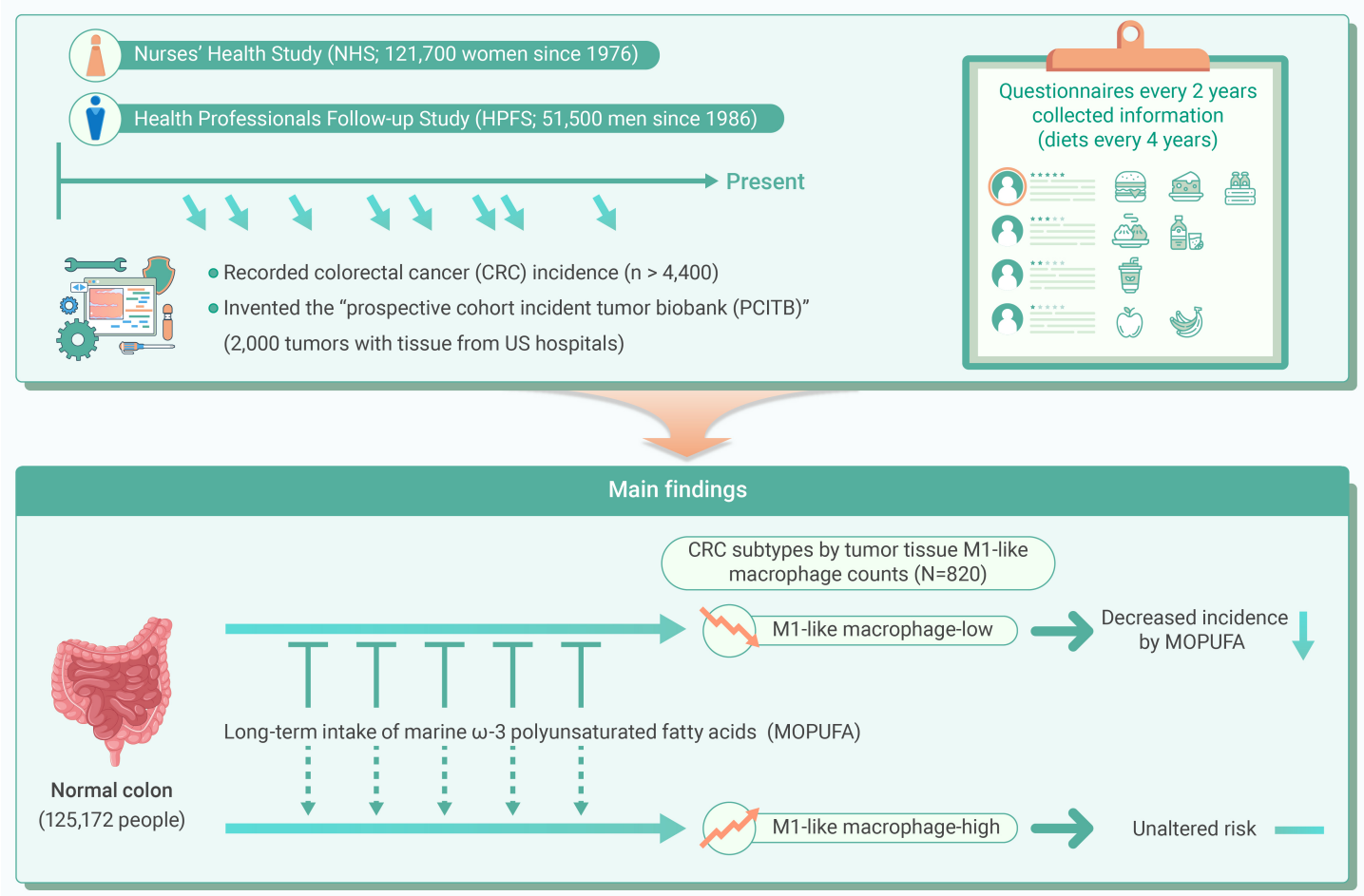
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- We assessed long-term intake of marine ω-3 polyunsaturated fatty acid (MOPUFA) and colorectal cancer (CRC) incidence.
- Higher MOPUFA intake correlated with lower incidence of CRC with scanty M1 macrophages (but not CRC with abundant M1 macrophages).
- The inverse association of MOPUFA intake with CRC incidence was stronger for tumors containing fewer M1 macrophages.
- MOPUFA may influence tumor-associated macrophages and CRC development.
- Dietary MOPUFA intake modifications may be used to improve cancer prevention.

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Evidence indicates that marine omega-3 polyunsaturated fatty acid (MOPUFA) intake exerts an immunomodulatory effect to suppress the development of colorectal cancer (CRC). We hypothesized that the association of MOPUFA intake with the incidence of CRC might differ by macrophage infiltrates in tumor tissue. We utilized the Prospective Cohort Incident Tumor Biobank within the Nurses' Health Study and Health Professionals Follow-up Study, both of which repeatedly assessed diets for decades of the follow-up of 125,172 participants, among whom 2,959 developed incident CRC. To spatially identify and count M1-polarized and M2-polarized macrophages in tumor tissue, we conducted *in situ* single-cell digital image analyses using multispectral immunofluorescence [for MRC1 (CD206), MAF, IRF5, CD86, and CD68] combined with machine learning algorithms. Using the 2,959 CRC cases, inverse probability weighting was integrated into the Cox proportional hazards models to adjust for tissue macrophage data availability in 820 cases. The multivariable-adjusted hazard ratio (with 95% confidence interval) for long-term MOPUFA intake of ≥0.25 g/day (vs. <0.15 g/day) were 0.56 (0.38-0.82; P_{trend} = 0.004) for the incidence of CRC with the lowest-quartile M1-like macrophage density. There was no significant association of MOPUFA intake with the incidence of CRC with the second to fourth quartile M1-like macrophage densities (P_{trend} > 0.20). The association of MOPUFA intake with CRC incidence differed by M1-like macrophages ($P_{heterogeneity}$ = 0.01), but not by M2-like macrophages. Our findings of the link between MOPUFA intake and lower incidence of CRC containing low M1-like macrophage counts provide evidence for differential influence of MOPUFAs on colorectal tumors with varying immune microenvironmental features.

INTRODUCTION

Evidence indicates that marine omega-3 polyunsaturated fatty acids (MOPUFAs) may exert an immunomodulatory effect to suppress the development of colorectal cancer (CRC).[1,2] Clinical data suggest that MOPUFAs have differential anti-CRC activity depending on several host factors, tumor characteristics, histological phenotype, and molecular features.[3] For example, a previous study showed differential associations of MOPUFA intake with CRC incidence by microsatellite instability (MSI) status, which is an important molecular biomarker that predicts response to immunotherapy in various cancer types.[4-6] These findings emphasize that subgrouping tumors based on key biological characteristics is an effective approach to elucidating mechanisms behind the protective effect of MOPUFAs on CRC development.

Macrophages are myeloid-derived immune cells and have heterogeneous and plastic phenotypes that are modified by the local tissue microenvironment.[7,8] Macrophages represent an essential component of the tumor immune microenvironment and often acquire divergent phenotypes, such as proinflammatory M1-like and anti-inflammatory M2-like functional polarizations, thereby exhibiting a variety of immunoregulatory and effector functions.[9-14] M1-like macrophages produce pro-inflammatory cytokines and exhibit antitumor activity, whereas M2-like macrophages secrete immunosuppressive cytokines and may promote tumor progression.[15,16] Considering evidence on the immunomodulatory effect of MOPUFAs, it is conceivable that MOPUFAs exert effect on the tumor immune microenvironment, thereby influencing tumorigenesis.

We therefore hypothesized that long-term MOPUFA intake might differentially influence CRC incidence by macrophage infiltrates in the tumor microenvironment. To identify and count M1-polarized and M2-polarized tumor-associated macrophages, we conducted spatial *in situ* single-cell digital image analyses using multispectral immunofluorescence combined with machine learning algorithms. We leveraged the recourses of two U.S.-wide longitudinal large-scale prospective cohort studies with repeated dietary assessments and integrated the immuno-pathological analyses into large-scale population research, to discover a link between long-term MOPUFA intake and CRC incidence subclassified by macrophage infiltrates and gain unique insight into the anti-tumor effect of MOPUFAs.

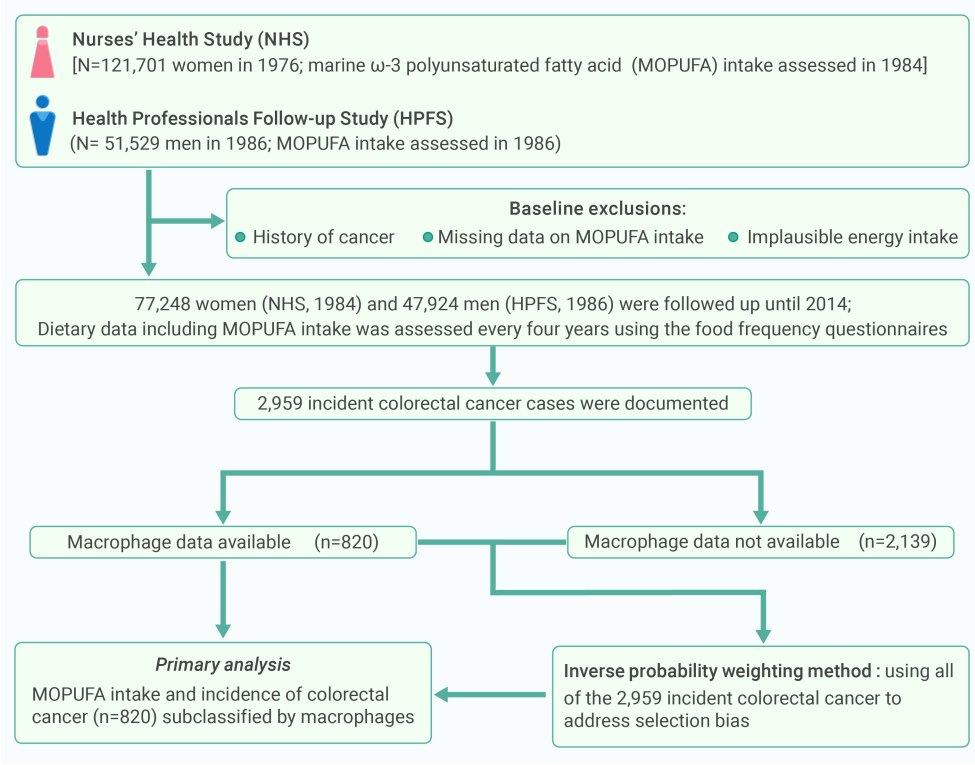

Figure 1. Flow chart of the study population
Abbreviations: MOPUFA, marine ω-3 polyunsaturated fatty acid.

MATERIALS AND METHODS

Study population

This study included participants from two U.S.-wide prospective cohort studies: namely, the Health Professionals Follow-up Study (HPFS) and Nurses' Health Study (NHS) (Figure 1). The details of these cohorts were described in our previous paper.[17] Briefly, the NHS and HPFS followed 121,701 female nurses and 51,529 male health professionals, respectively, for more than 30 years. In the HPFS and NHS, participants reported their lifestyles and vital status every two years, and their dietary habits using the food frequency questionnaires (FFQs) nearly every four years. Among participants who returned the baseline FFQs (in 1984 for the NHS; in 1986 for the HFPS), we excluded the following participants: 1) those who had >70 blank responses on the baseline FFQ, 2) those who had a history of cancer (except for non-melanoma skin cancer, 3) those who reported nearly implausible data (i.e., energy intake: <600 kcal/day or >3500 kcal/day for women, <800 kcal/day or >4200 kcal/day for men). We excluded participants who had a history of cancer (except for non-melanoma skin cancer) because 1) a substantial proportion of cancer patients had an advanced disease, which could affect exposures and distort the relationship between exposures and cancer risk, and 2) non-CRC cancer patients might develop metastatic cancers to the colorectum at any time in their lives. After these exclusions, 125,172 participants (47,924 men and 77,248 women) were eligible for the current analyses. Each participant provided informed consent at enrollment and additional consent for tissue use (from each CRC patient or if deceased, next-of-kin). This study was approved by the institutional review boards (IRBs) of the Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital (2019P003588).

Dietary and lifestyle assessments

Methods to measure MOPUFA intake have been described in our previous paper.[4] Using diet diaries and plasma nutrient measures, the FFQ method was extensively validated and showed good reproducibility in assessing various nutrients.[18,19] Briefly, self-reported dietary information was collected at baseline and updated almost every 4 years thereafter using the FFQ in the HPFS/NHS. For assessments of long-term dietary intake, repeated measurements using FFQ were shown to be superior to detailed diet records over short periods.[17] MOPUFA intake was the sum of the following: docosapentaenoic acid, eicosapentaenoic acid, and docosahexaenoic acid intake. The average daily intake was calculated by summing all intakes from foods and/or supplements (including fish oil) and adjusted for total caloric intake by the nutrient residual method.[20] The cumulative average MOPUFA intake at each FFQ cycle was calculated by averaging MOPUFA intake levels in all preceding (and then-current) FFQ cycles. Anthropometric, lifestyle, and medical factors in both cohorts were assessed, as previously described.[4]

Ascertainment of CRC cases

In both cohorts, incident CRC cases were reported by participants upon biennial questionnaire returns. Unreported lethal CRC were identified using the National Death Index. All incident CRC cases were confirmed through medical record review by study physicians. Up to 2014, we documented 2,959 incident CRC cases. Formalin-fixed paraffin-embedded (FFPE) tissue specimens were retrieved from hospitals and pathology laboratories throughout the United States, which led to the invention of the "Prospective Cohort Incident Tumor Biobank (PCITB)". The study pathologist (S.O.) examined hematoxylin and eosin-stained tissue sections and recorded histopathological features. Tissue microarrays within the PCITB were constructed using cases with adequate tumor tissue materials, as previously described.[4] Two to four tissue cores from each tumor were built in each tissue microarray block for downstream analyses.

Tumor tissue analyses

There was no established standard methods for the identification of M1 or M2 macrophages in FFPE tissue. To spatially identify and count M1-polarized and M2-polarized macrophages in the CRC microenvironment, we conducted spatial *in situ* single-cell digital image analyses using a multispectral immunofluorescence assay combined with machine learning algorithms (Figures 2-3), as previously described.[11] The multiplex panel contained antibodies for the following proteins: 1) CD68: a pan-macrophage marker, 2) CD86 and IRF5: M1 macrophage-associated markers, 3) MAF, MRC1 (CD206): M2 macrophage-associated markers, 4) KRT (keratin, cytokeratin): an epithelial cell marker (in addition to DAPI: a nuclear marker), according to the protein nomenclature recommendation by an expert panel.[21] Using the Vectra 3.0 multispectral imaging system (Akoya Biosciences, Marlborough, MA, USA), we scanned the tissue microarray slides with a 20× objective. To perform tissue category segmentation (tumor epithelial regions and stromal regions), cell segmentation, and cell type classification, the scanned images were processed with our pathologist-supervised machine learning algorithms within the inForm software package (Akoya Biosciences). For each macrophage, we evaluated the M1:M2 polarization index using the formula ([IRF5 × CD86] / [MAF × MRC1]), with each gene product symbol indicating its expression level.[11] Higher (or lower) M1:M2 index values indicated stronger M1-like (or M2-like) phenotype. The highest 30% of the index were defined as M1-like macrophages and the lowest 30% were defined as M2-like macrophages. A cell density (cell count per mm^2) was calculated in tumor stromal and epithelial regions separately.

We assessed tumor MSI status using DNA extracted from tumor and normal tissue specimens and the PCR assay for 10 microsatellite markers, as previously described.[22] MSI-high was defined as ≥30% of markers showing instability.

Statistical analysis

All statistical analyses were conducted by R Statistical Software (v4.1.2)

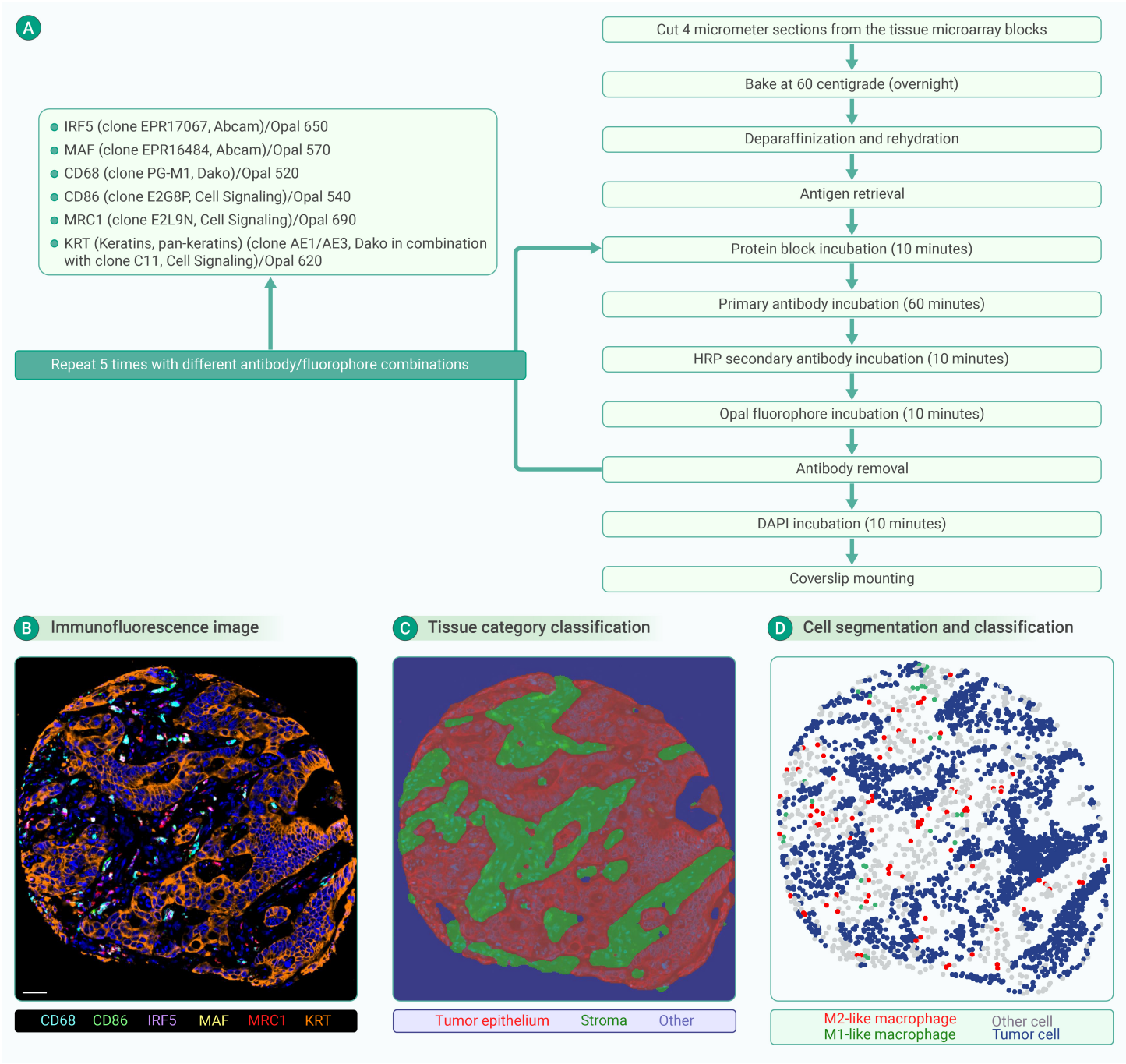

Figure 2. Evaluation of M1-like and M2-like macrophage infiltrates in CRC (A) Immunofluorescence procedure. (B-D) Examples of image analysis. To conduct tissue and cell segmentations and cell type classification, the tumor tissue images were processed with digital image analyses and pathologist-supervised machine learning algorithms using the inForm software package (Akoya Biosciences). Image data were further analyzed with R programming language. Macrophages were subclassified into M1-like and M2-like macrophage phenotypes based on an M1:M2 polarization index. The scale bar represents 50 μm. Detailed methods are described in Vayrynen et al.[11]

and SAS 9.4 (SAS Institute, Cary, NC, USA). Our primary hypothesis was that the association of MOPUFA intake with CRC incidence significantly differed by macrophage infiltrates in tumor. All other assessments represented secondary analyses. Detailed statistical analysis methods were described in Supplementary Materials. Briefly, the multivariable duplication method cause-specific Cox proportional hazards models were used to calculate hazard ratio (HR) for the incidence of each tumor subtype.[23] The Cox models were stratified by age, sex (except for sex-specific analyses), and year of questionnaire return. The multivariable Cox models were adjusted for potential confounders described in the footnotes in Tables. To adjust for potential selection bias from the availability of tissue macrophage data, we incorporated the inverse probability weighting (IPW) method using data of all of the 2,959 CRC patients into the Cox models.[23,24] The statistical trend test was performed using MOPUFA intake as a raw continuous variable except for individuals in the 5th sex-specific highest quintile, for whom the median of the 5th sex-specific quintile values was used to eliminate outlier effects. To examine statistical heterogeneity across the ordinal tumor subtypes (i.e., quartile macrophage subtypes), we applied the meta-regression method.[25]

We used two-sided *P* values, and considered *P* values < 0.005 as statistically significant, as recommended by experts in statistics.[26] *P* values between 0.05 and 0.005 were interpreted as suggestive evidence.

RESULTS

Characteristics of study participants

Table 1 shows age-standardized characteristics according to MOPUFA intake. Participants with higher MOPUFA intake tended to have higher physi-

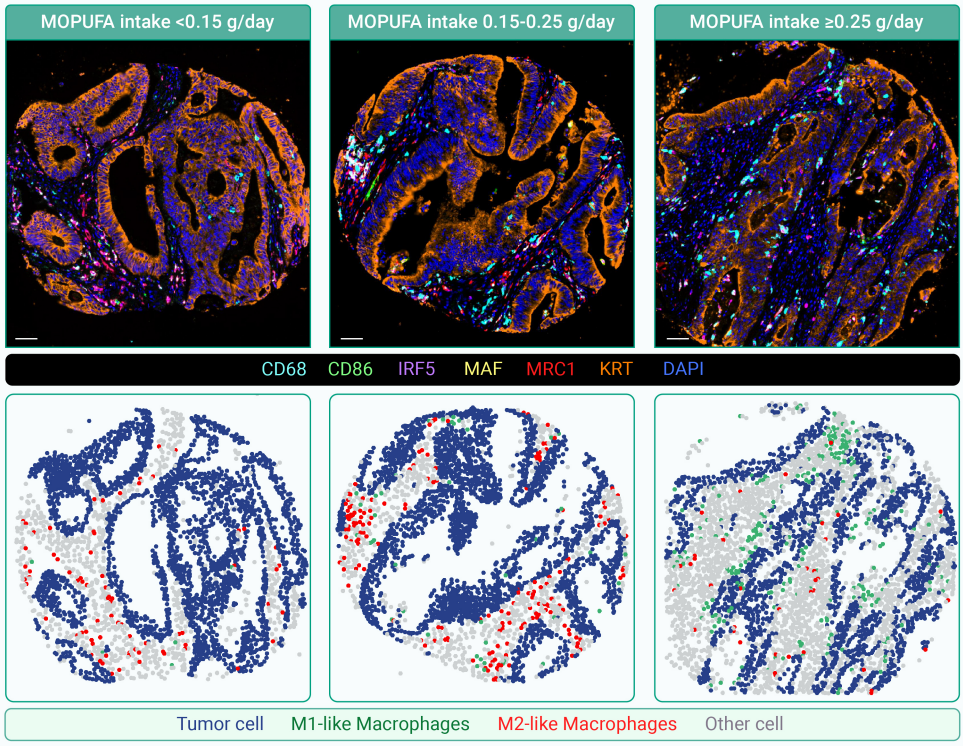

Figure 3. Quantitative, multispectral evaluation of M1-like and M2-like macrophage markers in the colorectal cancer microenvironment Representative immunofluorescence images of colorectal carcinomas with low (< 0.15 g/day), intermediate (0.15-0.25 g/day), and high (≥ 0.25 g/day) marine ω-3 polyunsaturated fatty acid (MOPUFA) intake. The scale bar represents 50 μm.

cal activity, undergo endoscopy, use multivitamins, and consume higher amounts of calcium, folate, vitamin D, and fiber, and eat less red meat.

Colorectal cancer incidence during follow-up and spatial single-cell tissue analyses

During the prospective follow-up of the 125,172 participants, 2,959 individuals developed histopathologically confirmed CRC. Among these CRC cases, we conducted spatial *in situ* single-cell analyses using multispectral immunofluorescence combined with machine learning algorithms to identify and count M1-like and M2-like tumor-associated macrophages in 820 CRC cases within the Prospective Cohort Incident Tumor Biobank (PCITB) (Figures 2-3). There was no substantial difference in the characteristics between CRC cases with and without tumor macrophage data (Table S1). The association of MOPUFA intake with CRC incidence did not substantially differ between the two CRC case groups (i.e., cases with tumor macrophage data and those without tumor macrophage data) (Table S2). To adjust for potential selection bias due to tumor macrophage data availability, we incorporated the inverse probability weighting (IPW) method into the Cox models.[20,21]

MOPUFA intake and CRC incidence by M1-like macrophages

We classified the 820 CRC cases into quartile categories of M1-like (or M2-like) macrophage density (cell count divided by tissue area) in epithelial, stromal, or combined tissue areas. We tested our primary hypothesis of a differential association of MOPUFA intake with CRC incidence classified by macrophage densities (Table 2). We observed such a difference in terms of M1-like macrophages in combined epithelial and stromal areas ($P_{heterogeneity}$ = 0.01, across M1-like macrophage categories). Higher MOPUFA intake was associated with lower incidence of CRC having the lowest quartile M1-like macrophage density, with the multivariable hazard ratio (HR; for MOPUFA intake of ≥0.25 g/day vs. <0.15 g/day) of 0.56 [95% confidence interval (CI), 0.38 to 0.82; P_{trend} = 0.004). In contrast, MOPUFA intake was not associated with the incidence of CRC containing higher-level (the second to fourth quartiles) M1-like macrophage density (P_{trend} > 0.20). The inverse associations of MOPUFA intake with CRC incidence were also observed for the lowest quartile of M1-like macrophage density in epithelial (P_{trend} = 0.04) or stromal areas (P_{trend} = 0.004) (Tables S3-4).

We did not observe significant heterogeneity in the association of MOPUFA intake with CRC incidence subclassified by M2-like macrophages ($P_{heterogeneity}$ > 0.20) (Tables 2 & S3-4).

Considering previous evidence on differential associations of MOPUFA intake with CRC subclassified by MSI status,[4] we conducted stratified analyses by MSI status (Table 3). The inverse association of MOPUFA intake with non-MSI-high CRC was stronger for tumors containing lower level M1-like macrophages. Multivariable-adjusted HRs for MOPUFA intake of ≥0.25 g/day (vs. <0.15 g/day) were 0.56 (95% CI, 0.37 to 0.85; P_{trend} = 0.005) for non-MSI-high CRC with the lowest quartile of M1-like macrophage density. Statistical power was limited in the analyses for MSI-high CRC.

We conducted sensitivity analyses without IPW adjustment. Similar to IPW-adjusted analyses, we observed a differential association of MOPUFA with CRC incidence classified by M1-like macrophage infiltrates ($P_{heterogeneity}$ = 0.04; Table S5).

DISCUSSION

CRC is a group of complex multifactorial chronic diseases with long-term influence of diet, lifestyle, and environment.[11,27] Therefore, integrative analyses of tumor biomarkers into large population data have been developed to decipher the influences of exogenous factors on tumor characteristics in humans.[28-30] Leveraging the resources of large-scale prospective cohort studies, we found that high intake of MOPUFA was associated inversely with the incidence of CRC containing scanty M1-like macrophages but not that of CRC containing abundant M1-like macrophages. Notably, we confirmed that such differential associations were consistently observed in the non-MSI-high CRC stratum.

The tumor microenvironment is an essential component of neoplasm.[10,28,31] While some studies have reported that a high infiltration of macrophages into colorectal tumors is associated with better patient prognosis,[32-34] others have shown contradictory findings.[35-37] These conflicting results may be related to the heterogeneity of macrophage populations and measurement methods.[9,10] We also recognize that any of individual cellular markers are not specific to M1 or M2 macrophages.[38] To address this challenge in evaluating macrophage polarization phenotypes, we developed and validated our multispectral immunofluorescence assay.[11] This approach enabled more detailed evaluation of macrophage polarization compared with single or double marker approaches. In fact, we showed that M1-like and M2-like macrophages in CRC were divergently associated with better and poor patient prognosis, respectively.[11]

The present study showed the strong inverse association of MOPUFA intake with the incidence of CRC containing low M1-like macrophage counts. Epidemiological evidence indicates that fish or MOPUFA intake may be associated with reduced risk of several cancer types, including CRC, liver cancer, breast cancer, prostate cancer, and brain tumor.[39] Experimental studies have shown that MOPUFAs can alter gene expression profiles in macrophages and inhibit the production of cytokines to reduce inflammation.[40-42] Among MOPUFAs, docosahexaenoic acid and eicosapentaenoic acid have been shown to enhance the phagocytic capacity of macrophages.[43,44] Moreover, dietary MOPUFAs have been found to substantially increase tumor infiltration of M1-polarized macrophages and decrease infiltration of M2-polarized macrophages in a mouse model, which provides a potential mechanistic

Table 1. Age-standardized characteristics according to MOPUFA intake

Characteristics*	NHS (women, 1984-2014)			HPFS (men, 1986-2014)		
	MOPUFA intake‖ (g/day)			MOPUFA intake‖ (g/day)		
	< 0.15	0.15 – 0.25	≥ 0.25	< 0.15	0.15–0.25	≥ 0.25
Person-years	729,594	623,004	721,568	262,331	250,102	594,666
Mean age (years)	62.1	63.6	64.1	62.4	64.0	65.4
Mean body mass index (kg/m^2)	25.6	25.8	26.0	25.9	25.8	25.8
Mean physical activity (METS-hours/week)	14.0	16.3	19.3	23.9	26.4	29.8
Mean pack-years smoked	13.2	12.7	12.5	13.0	12.5	11.1
Colorectal cancer in a parent or sibling, %	19.5	19.6	19.8	14.4	14.9	14.9
History of previous endoscopy, %	24.7	26.7	28.8	23.8	26.7	30.2
Regular aspirin or NSAID use, %‡	50.7	52.8	52.2	43.9	47.6	48.4
Postmenopausal, %	81.7	82.9	83.4			
Current hormone use, %§	45.9	45.9	44.3			
Dietary intake						
Omega-3 PUFAs (g/day)	1.13	1.23	1.45	1.20	1.33	1.65
18:3 omega -3 (ALA) (g/day)	0.95	0.96	1.00	1.09	1.10	1.12
20:5 omega -3 (EPA)‖ (mg/day)	26	58	129	25	60	166
22:5 omega -3 (DPA)‖ (mg/day)	14	19	30	15	22	39
22:6 omega -3 (DHA)‖ (mg/day)	59	117	229	53	116	274
Omega -6 PUFAs (g/day)	8.17	8.14	7.93	12.11	11.93	11.60
Fish (servings/week)	0.9	1.7	3.1	0.7	1.5	3.3
Total red meat (servings/week)	6.3	5.9	4.9	7.7	7.2	5.4
Alcohol (g/day)	5.2	6.3	6.5	10.1	11.5	11.2
Folate (μg/day)	417	449	502	487	516	588
Calcium (mg/day)	989	1035	1113	923	921	951
Vitamin D (IU/day)	317	354	440	342	381	501
Total fiber (g/day)	16.7	17.7	19.0	20.4	21.1	23.3

* To calculate the mean or percentage for variables, we used updated information throughout follow-up. Except for age, all variables are age standardized. ‖ MOPUFA intake is the sum of consumptions of EPA, DPA, and DHA. ‡ We defined regular aspirin or NSAID use as ≥2 standard tablets of aspirin or ≥2 tablets of NSAIDs per week. § Percentages of current menopausal hormone use was calculated only among postmenopausal women. Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HPFS, Health Professionals Follow-up Study; METS, metabolic equivalent task score; MOPUFA, marine ω-3 polyunsaturated fatty acid; NHS, Nurses' Health Study; NSAID, non-steroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid.

explanation for inhibitory effects on tumor development and progression.[45,46]

Tumor molecular features have profound effects on anti-tumor immunity and response to immunotherapy.[47] In particular, the MSI-high status can influence immune response to CRC, and there is substantial tumor heterogeneity between MSI-high and non-MSI-high CRCs.[47,48] Notably, a previous study showed that the inverse association of MOPUFAs with CRC incidence was confined to MSI-high tumors.[4] In our analyses, the association of MOPUFA intake with lower incidence of CRC containing fewer M1-like macrophages was observed regardless of the MSI status. Our findings suggest that the potential cancer prevention effect of MOPUFAs through M1 macrophages might be independent of MSI status. Evidence on the impact of MOPUFA intake on immunotherapy is scarce. A pilot study reported a higher serum level of eicosapentaenoic acid in the long-term survivors compared to the short-term survivors among non-small cell lung cancer patients who received an immune checkpoint inhibitor.[49] Other studies showed that MOPUFA intake after CRC diagnosis was associated with lower risk of recurrence and CRC-specific mortality in CRC patients.[50,51] These studies support the potential of the adjuvant nutritional intervention with MOPUFAs for enhancing the efficacy of treatment (including immunotherapy) for CRC.

Some limitations of the present study should be noted. First, measurement errors were inevitably present in the questionnaire-based assessments of dietary factors as well as the quantifications of the macrophage densities. Nonetheless, these measurement errors would most likely drive our results towards null findings. In addition, we conducted rigorous validation in tumor-associated macrophage measurements.[11] Second, residual and unmeasured confounding could be present in our observational study, although we adjusted for a comprehensive set of potential confounders in our multivariable models. Third, the data on macrophages were not available for all CRC patients. However, we utilized the IPW method to adjust for selection bias due to tissue availability. We observed similar results in both IPW-adjusted and non-IPW-adjusted analyses. Fourth, the study population mostly consisted of non-Hispanic Caucasians. Therefore, our results should be validated in other populations.

Our study based on the PCITB has several strengths. First, we prospec-

Table 2. MOPUFA intake and incidence of colorectal cancer overall and by macrophage density in combined tissue areas

	MOPUFA intake, g/day			P_{trend}*	$P_{heterogeneity}$†
	< 0.15	0.15–0.25	≥ 0.25		
Person-years	991,925	873,106	1,316,234		
Overall colorectal cancer					
Cases (n=820)	268	233	319		
Age-adjusted HR (95% CI)§	1 (referent)	1.00 (0.91-1.11)	0.79 (0.72-0.87)	<0.001	
Multivariable HR (95% CI)‖	1 (referent)	0.99 (0.90-1.10)	0.81 (0.73-0.89)	<0.001	
M1-like macrophage density					0.011
Quartile 1					
Cases (n=205)	78	64	63		
Age-adjusted HR (95% CI)§	1 (referent)	1.09 (0.76-1.57)	0.55 (0.37-0.81)	0.003	
Multivariable HR (95% CI)‖	1 (referent)	1.06 (0.74-1.51)	0.56 (0.38-0.82)	0.004	
Quartile 2					
Cases (n=205)	68	55	82		
Age-adjusted HR (95% CI)§	1 (referent)	0.97 (0.66-1.41)	0.85 (0.60-1.21)	0.17	
Multivariable HR (95% CI)‖	1 (referent)	0.97 (0.66-1.42)	0.87 (0.61-1.23)	0.20	
Quartile 3					
Cases (n=205)	57	68	80		
Age-adjusted HR (95% CI)§	1 (referent)	1.44 (0.99-2.09)	1.05 (0.73-1.51)	0.55	
Multivariable HR (95% CI)‖	1 (referent)	1.40 (0.97-2.04)	1.07 (0.75-1.54)	0.47	
Quartile 4					
Cases (n=205)	65	46	94		
Age-adjusted HR (95% CI)§	1 (referent)	0.64 (0.43-0.97)	0.85 (0.60-1.19)	0.44	
Multivariable HR (95% CI)‖	1 (referent)	0.65 (0.43-0.98)	0.87 (0.62-1.23)	0.54	
M2-like macrophage density					0.29
Quartile 1					
Cases (n=205)	63	58	84		
Age-adjusted HR (95% CI)§	1 (referent)	1.09 (0.73-1.63)	0.89 (0.61-1.31)	0.69	
Multivariable HR (95% CI)‖	1 (referent)	1.08 (0.73-1.61)	0.91 (0.63-1.33)	0.76	
Quartile 2					
Cases (n=205)	59	67	79		
Age-adjusted HR (95% CI)§	1 (referent)	1.23 (0.85-1.80)	0.86 (0.59-1.25)	0.75	
Multivariable HR (95% CI)‖	1 (referent)	1.21 (0.84-1.76)	0.86 (0.60-1.25)	0.80	
Quartile 3					
Cases (n=205)	70	55	80		
Age-adjusted HR (95% CI)§	1 (referent)	0.92 (0.62-1.35)	0.73 (0.52-1.03)	0.050	
Multivariable HR (95% CI)‖	1 (referent)	0.90 (0.61-1.33)	0.75 (0.53-1.05)	0.067	
Quartile 4					
Cases (n=205)	76	53	76		
Age-adjusted HR (95% CI)§	1 (referent)	0.80 (0.55-1.16)	0.70 (0.50-0.99)	0.040	
Multivariable HR (95% CI)‖	1 (referent)	0.79 (0.55-1.15)	0.72 (0.51-1.02)	0.057	

* Trend test was performed using MOPUFA intake as a raw continuous variable except for individuals in the 5th sex-specific quintile for whom the median of the 5th sex-specific quintile values was used in the model to mitigate outlier effects. † Meta-regression method with a subtype-specific random effect term was applied to examine statistical heterogeneity between tumor subtypes. § Inverse probability weighted Cox proportional hazards model was used with stratification by age, sex (i.e., cohort), and calendar year of questionnaire cycles. ‖ Additionally adjusted for alcohol consumption (continuous with 30 g/day ceiling), regular use of aspirin or nonsteroidal anti-inflammatory drugs (>2 tablets/week; yes or no), physical activity (continuous with a ceiling at 50 METS-hours per week), previous lower gastrointestinal endoscopy (yes or no), family history of colorectal cancer in any first-degree relative (yes or no), cumulative pack-years smoked (continuous with 50 pack-years ceiling), and body mass index (continuous with 35 kg/m^2 ceiling). Abbreviations: CI, confidence interval; HR, hazard ratio; METS, metabolic equivalent task score; MOPUFA, marine ω-3 polyunsaturated fatty acid.

Table 3. Incidence of colorectal cancer, jointly subclassified by M1-like macrophage density (combined tissue areas) and MSI status, in relation to MOPUFA intake

	MOPUFA intake (g/day)			P_{trend}*
	< 0.15	0.15-0.25	≥ 0.25	
Person-years	991,925	873,106	1,316,234	
Microsatellite instability (MSI) status				
Non-MSI-high				
M1-like macrophage density quartiles				
Quartile 1				
Cases (n=181)	66	58	57	
Age-adjusted HR (95% CI)§	1 (referent)	1.10 (0.74-1.62)	0.55 (0.36-0.84)	0.005
Multivariable HR (95% CI)‖	1 (referent)	1.07 (0.73-1.57)	0.56 (0.37-0.85)	0.005
Quartile 2				
Cases (n=179)	57	50	72	
Age-adjusted HR (95% CI)§	1 (referent)	1.03 (0.69-1.54)	0.85 (0.58-1.24)	0.19
Multivariable HR (95% CI)‖	1 (referent)	1.04 (0.69-1.56)	0.86 (0.59-1.26)	0.22
Quartile 3				
Cases (n=158)	44	51	63	
Age-adjusted HR (95% CI)§	1 (referent)	1.23 (0.82-1.84)	1.03 (0.69-1.54)	0.60
Multivariable HR (95% CI)‖	1 (referent)	1.19 (0.79-1.80)	1.06 (0.71-1.59)	0.50
Quartile 4				
Cases (n=145)	43	34	68	
Age-adjusted HR (95% CI)§	1 (referent)	0.67 (0.41-1.10)	0.96 (0.63-1.45)	0.92
Multivariable HR (95% CI)‖	1 (referent)	0.69 (0.42-1.11)	1.00 (0.66-1.51)	0.93
MSI-high				
M1-like macrophage density quartiles				
Quartile 1				
Cases (n=20)	12	4	4	
Age-adjusted HR (95% CI)§	1 (referent)	0.80 (0.25-2.54)	0.43 (0.14-1.32)	0.32
Multivariable HR (95% CI)‖	1 (referent)	0.77 (0.25-2.42)	0.45 (0.14-1.42)	0.39
Quartile 2				
Cases (n=23)	11	3	9	
Age-adjusted HR (95% CI)§	1 (referent)	0.29 (0.08-1.12)	0.66 (0.26-1.69)	0.32
Multivariable HR (95% CI)‖	1 (referent)	0.29 (0.07-1.10)	0.67 (0.27-1.70)	0.31
Quartile 3				
Cases (n=35)	12	11	12	
Age-adjusted HR (95% CI)§	1 (referent)	1.57 (0.65-3.78)	0.95 (0.37-2.44)	0.91
Multivariable HR (95% CI)‖	1 (referent)	1.53 (0.64-3.64)	0.94 (0.37-2.40)	0.90
Quartile 4				
Cases (n=55)	21	12	22	
Age-adjusted HR (95% CI)§	1 (referent)	0.63 (0.29-1.35)	0.62 (0.32-1.17)	0.15
Multivariable HR (95% CI)‖	1 (referent)	0.63 (0.29-1.37)	0.64 (0.34-1.20)	0.17

* Trend test was performed using MOPUFA intake as a raw continuous variable except for individuals in the 5th sex-specific quintile for whom the median of the 5th sex-specific quintile values was used in the regression model to mitigate outlier effects. § Inverse probability weighted Cox proportional hazards model was used with stratification by age, sex (i.e., cohort), and calendar year of current questionnaire cycle. ‖ Additionally adjusted for alcohol consumption (continuous with 30 g/day ceiling), regular use of aspirin or nonsteroidal anti-inflammatory drugs (>2 tablets/week; yes or no), physical activity (continuous with a ceiling at 50 METS-hours per week), previous lower gastrointestinal endoscopy (yes or no), family history of colorectal cancer in any first-degree relative (yes or no), cumulative pack-years smoked (continuous with 50 pack-years ceiling), and body mass index (continuous with 35 kg/m^2 ceiling). Abbreviations: CI, confidence interval; HR, hazard ratio; MOPUFA, marine omega-3 polyunsaturated fatty acid; MSI, microsatellite instability.

tively and repeatedly collected data on MOPUFA intake every 4 years during the 30-year follow-up period. This study design enabled us to not only estimate long-term cumulative average intake, but also to eliminate differential recall bias between incident CRC patients and cancer-free individuals. Second, the prospective cohort design also enabled us to adjust for selection bias due to tissue availability using the IPW-adjusted models constructed by data of all incident CRCs in the cohorts. Third, *in situ* single-cell analyses with multispectral immunofluorescence were used to identify and quantify M1-like and M2-like macrophages, which could provide a more granular spatial view of cell phenotypes than conventional single-color or double-color immunohistochemistry. Notably, there has been no established standardized method to assess the M1:M2 polarization of macrophages in archival tissue. Hence, we used this robust multispectral technology to identify M1-like and M2-like macrophages. Fourth, our CRC cases were derived from hospitals throughout the United States, which increased the generalizability of the findings.

In conclusion, we showed that MOPUFA intake was associated with lower incidence of CRC containing low M1-like macrophage counts (but not CRC containing high M1-like macrophage counts), providing evidence for the cancer prevention effect of MOPUFAs through the modulation of the tumor-immune microenvironment.

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AUTHOR CONTRIBUTIONS

S.O. developed the main concept and designed the study. T.U., M.S., A.T.C., J.A.M., and S.O. wrote grant applications. T.U., J.P.V., R.Z., S.U., K.H., M.G., J.A.M., J.A.N., and S.O. were responsible for collection of tumor tissue, and acquisition of epidemiologic, clinical and tumor tissue data. T.U., R.Z., S.U., and S.O. performed data analysis, interpreted the results, and drafted the manuscript. All authors contributed to editing and critical revision for important intellectual contents.

DECLARATION OF INTERESTS

J.L.G. serves as a consultant for GlaxoSmithKline, Codagenix, Array BioPharma, and Verseau Therapeutics; and receives research support from GlaxoSmithKline, Eli Lilly and Array BioPharma for the study of the breast tumor microenvironment. A.T.C. previously served as a consultant for Bayer Healthcare and Pfizer Inc.. M.G. receives research funding from Janssen, consulting fees from Nerviano Medical Sciences and has received honoraria from ChromaCode and AstraZeneca. C.S.F. previously served as a consultant for Agios, Bain Capital, Bayer, Celgene, Dicerna, Five Prime Therapeutics, Gilead Sciences, Eli Lilly, Entrinsic Health, Genentech, KEW, Merck, Merrimack Pharmaceuticals, Pfizer Inc, Sanofi, Taiho, and Unum Therapeutics; C.S.F. also serves as a Director for CytomX Therapeutics and owns unexercised stock options for CytomX and Entrinsic Health. C.S.F. is currently employed by Genentech, a subsidiary of Roche. J.A.M. has received institutional research funding from Boston Biomedical, has served as an advisor/consultant to Ignyta and COTA Healthcare, and served on a grant review panel for the National Comprehensive Cancer Network funded by Taiho Pharmaceutical. This study was not funded by any of these commercial entities.

ETHICAL STATEMENT AND PATIENT CONSENT

This study was approved by the institutional review boards (IRBs) of the Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital (2019P003588). Each participant provided informed consent at enrollmentand additional consent for tissue use (from each CRC patient or if deceased, next-of-kin).

USE OF STANDARDIZED OFFICIAL SYMBOLS

We use HUGO (Human Genome Organisation)-approved official symbols (or root symbol) for genes and gene products, including CD68, CD86, IRF5, KRT, MAF, and MRC1; all of which are described at www.genenames.org/. Gene names are italicized, and protein names are non-italicized.

DATA AND CODE AVAILABILITY

The datasets generated and/or analyzed during the current study are not publicly available. Further information including the procedures to obtain and access data from the Nurses' Health Studies and the Health Professionals Follow-up Study are described at https://www.nurseshealthstudy.org/researchers/ and https://sites.sph.harvard.edu/hpfs/for-collaborators/.

SUPPLEMENTAL INFORMATION

It can be found online at https://doi.org/10.59717/j.xinn-med.2024.100082

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