

Evaluating Ultra-long-Chain Fatty Acids as Biomarkers of Colorectal Cancer Risk

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Abstract

Background: Cross-sectional studies reported a novel set of hydroxylated ultra-long-chain fatty acids (ULCFA) that were present at significantly lower levels in colorectal cancer cases than controls. Follow-up studies suggested that these molecules were potential biomarkers of protective exposure for colorectal cancer. To test the hypothesis that ULCFAs reflect causal pathways, we measured their levels in prediagnostic serum from incident colorectal cancer cases and controls.

Methods: Serum from 95 colorectal cancer patients and 95 matched controls was obtained from the Italian arm of the European Prospective Investigation into Cancer and Nutrition cohort and analyzed by liquid chromatography–high-resolution mass spectrometry. Levels of 8 ULCFAs were compared between cases and controls with paired *t* tests and a linear model that used time to diagnosis (TTD) to determine whether case–control differences were influenced by disease progression.

Results: Although paired *t* tests detected significantly lower levels of four ULCFAs in colorectal cancer cases, confirming earlier reports, the case–control differences diminished significantly with increasing TTD (7 days–14 years).

Conclusion: Levels of several ULCFAs were lower in incident colorectal cancer cases than controls. However, because case–control differences decreased with increasing TTD, we conclude that these molecules were likely consumed by processes related to cancer progression rather than causal pathways.

Impact: ULCFA levels are unlikely to represent exposures that protect individuals from colorectal cancer. Future research should focus on the diagnostic potential and origins of these molecules. Our use of TTD as a covariate in a linear model provides an efficient method for distinguishing causal and reactive biomarkers in biospecimens from prospective cohorts. *Cancer Epidemiol Biomarkers Prev*; 25(8): 1216–23. ©2016 AACR.

Introduction

Colorectal cancer accounts for one fourth of all cancer-related deaths worldwide and is the second leading cause of cancer mortality in the United States and Europe (1, 2). As less than 15 percent of the variation in risk of colorectal cancer has been attributed to heritable genetic factors (3, 4), exposures such as nutrients, microbial metabolites, toxins, and pathogens are likely to play a significant role in colorectal cancer development. Exposures that have been associated with increased risks of colorectal cancer include obesity, cigarette smoking, alcohol use, and consumption of n-6 polyunsaturated fatty acids, all of which contribute to oxidative stress and inflammation [reviewed in Stone and colleagues (5)]. On the other hand, regular consumption of aspirin, an antioxidant and anti-inflammatory drug, reduces colorectal cancer risk (5, 6). Aspirin

inhibits both COX-1 and COX-2 enzymes, preventing the production of inflammatory prostaglandins and thromboxanes (7) and also acetylates COX-2 and thereby allows conversion of n-3 and n-6 fatty acids to inflammation-resolving compounds (lipoxins are derived from n-6 fatty acids and resolvins and protectins from n-3 and n-6 fatty acids; ref. 8). This combination of factors suggests that colorectal cancer may result from an imbalance in production and removal of reactive electrophiles and inflammatory products that can initiate and promote tumors (5, 9, 10).

Recently, Ritchie and colleagues, used untargeted high-resolution mass spectrometry (HRMS) to detect a novel class of polyunsaturated, hydroxylated, ultra-long-chain fatty acids (ULCFA, containing between 28 and 36 carbons) that was associated with reduced risks of colorectal cancer in three case–control studies (11). Using accurate-mass signatures of a dozen representative ULCFAs, Ritchie and colleagues reported that concentrations of these molecules were not correlated with either the tumor stage or type of treatment in cases. Furthermore, ULCFA levels declined with increasing age (whereas risk of colorectal cancer increases with age) in cases and controls, indicating a possible protective effect of ULCFAs (12). Moreover, a large follow-up study of colonoscopy patients by the same authors indicated that subjects under the age of 50 that were in the lowest decile of ULCFA–serum concentrations had a relative colorectal cancer risk of 10.1 (CI: 6.4–16.4; ref. 13).

In attempting to elucidate a protective mechanism for these molecules, Ritchie and colleagues dosed human colorectal cancer (SW620) cells with 28-carbon ULCFAs that had been isolated

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from human serum, and reported reduced production of proinflammatory markers (NFKB2, NFKBIA, and NOS2; ref. 14). Since, as noted above, inflammation has been a hallmark of colorectal cancer (5, 9, 15), the inverse correlation of ULCSFA levels and colorectal cancer risk would be consistent with a cancer mechanism that favors a proinflammatory environment that increases with age. Furthermore, the purported anti-inflammatory or protective properties of ULCSFAs could be similar to those of hydroxylated very-long chain fatty acids that are metabolized into inflammation-resolving compounds (i.e., lipoxins, resolvins, and protectins). These compounds are active in the picomolar–nanomolar range (10) and have epimeric forms that are triggered by aspirin, which reduces risks of colorectal cancer and cancer generally (6, 16).

Remarkably, the provocative findings of Ritchie and colleagues (11–14, 17) implicating low serum levels of ULCSFAs as potential causes of colorectal cancer have not been explored by other investigators. As all of the reported associations between circulating levels of ULCSFAs and colorectal cancer were derived from cross-sectional studies (11) it is particularly important to replicate Ritchie's findings with archived cohort samples that were collected prior to colorectal cancer diagnosis. This would reduce the likelihood that lower levels of ULCSFAs in colorectal cancer cases resulted from tumor-induced dysregulation of homeostatic pathways (reverse causality). The purpose of this study is to test the hypothesis that ULCSFAs are potentially protective against colorectal cancer with prediagnostic serum from 95 incident colorectal cancer cases and matched controls from the European Prospective Investigation of Cancer and Nutrition (EPIC). Also, as previous reports had implicated consumption of seafood as being potentially protective of colorectal cancer (18, 19), several fresh seafood samples were tested for the presence of ULCSFAs.

Materials and Methods

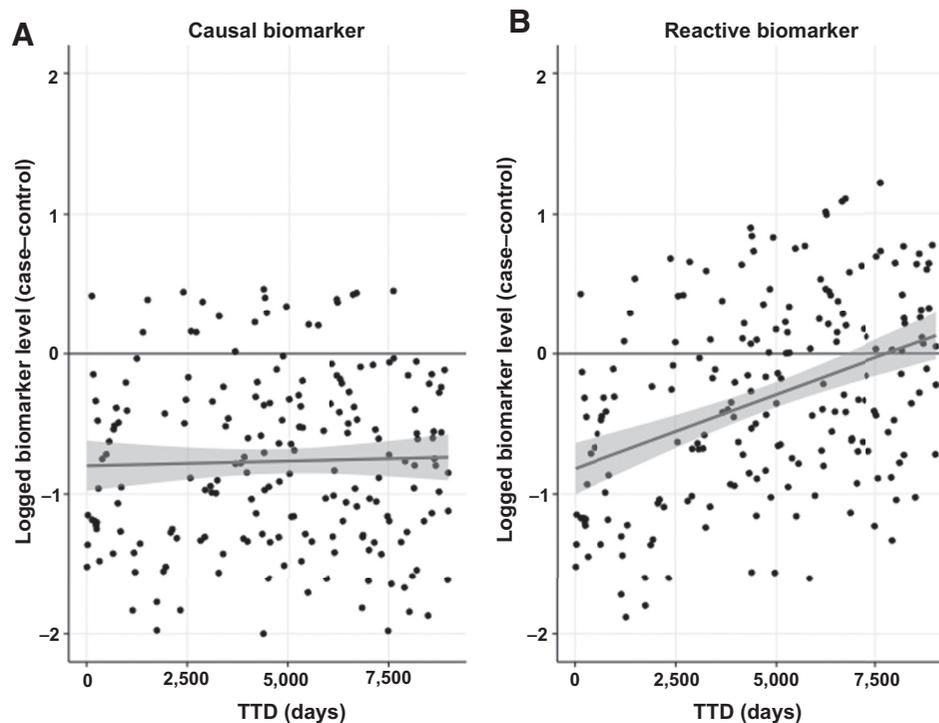
Experimental design

We adopted a simple regression model to determine whether ULCSFAs represent biomarkers on the causal pathway to colorectal cancer or are reactive biomarkers related to progression of the disease. As the EPIC serum had been obtained between 7 days and 14 years prior to colorectal cancer diagnosis, we used the (log-scale) difference in ULCSFA concentrations (colorectal cancer case minus matched control) as the outcome variable in a linear model to simultaneously investigate effects of case status and time to diagnosis (TTD) on the risk of colorectal cancer (note that these log-scale case–control differences represent case:control ratios in natural scale). The model is shown as follows:

$$Y_i = \beta_0 + \beta_1(TTD)_i + \varepsilon_i, \quad (1)$$

where Y_i represents the case–control difference of (log-transformed) ULCSFA levels for the i^{th} case–control pair, β_0 is the intercept representing the case–control difference at recruitment, and β_1 is the coefficient for TTD (d). Evidence favoring a non-zero intercept (β_0) would indicate that a given ULCSFA level differed on average between cases and controls. A negative intercept, illustrated with the hypothetical example in Fig. 1A, would indicate higher ULCSFA levels in controls (i.e., a protective effect) as suggested by Ritchie and colleagues (11). Likewise, a significant coefficient for TTD (β_1), illustrated in Fig. 1B, would indicate that the timing of blood collection relative to diagnosis affected the outcome and, therefore, that any case–control difference in the ULCSFA level probably reflects progression of colorectal cancer. Thus, the combination of a negative β_0 and nonsignificant β_1 would point to a potentially causal

Figure 1. Use of a linear model (model 1) to differentiate a causal biomarker from a disease-related biomarker. Hypothetical data representing levels of a biomarker were generated for case–control pairs, transformed to natural logarithms, normalized to zero mean, and the case–control differences plotted versus time to diagnosis (TTD). **A**, case–control differences are consistently less than zero indicating that biomarker levels are greater in controls than in cases and are not affected by TTD. This would indicate a biomarker of protective effect. **B**, case–control differences diminish with increasing TTD, consistent with a biomarker of disease progression.



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biomarker of colorectal cancer while a significant β_1 would point to a reactive biomarker.

Study population

EPIC is a large prospective cohort study with approximately 520,000 participants, ages 25–70 years at enrollment from 1992 through 2000, from 23 centers in 10 European countries (20). All study participants provided written informed consent. Serum was collected at enrollment and dietary information was obtained with a food-frequency questionnaire (21, 22). The serum for this investigation consisted of 190 specimens (95 case–control pairs), collected between 1993 and 1997 from subjects in Turin, Italy. Controls were matched to incident cases by age, study enrollment year and season, and gender. Summary statistics for these subjects are listed in Table 1 including TTD, gender, body mass index (BMI), waist circumference, and self-reported consumption of fish and shellfish. These covariates were selected on the basis of previous evidence that BMI and waist circumference are associated with colorectal cancer risk (23, 24) and that diets rich in fish oil have reduced risks of inflammation-related diseases (18, 19).

Chemicals

LC-MS grade (Fluka) isopropanol, methanol, water, and ^{13}C -cholic acid (internal standard) were from Sigma-Aldrich. LC-MS grade (Optima) acetic acid and chloroform were from Fisher Scientific. All chemicals were of analytic grade and were used without purification.

Sample processing

Shortly after collection, a 0.5-mL aliquot of each serum sample was placed in a cryostraw, sealed, and stored in liquid nitrogen (-196°C) at the International Agency for Research on

Cancer in Lyon, France. Approximately one year prior to analysis, cryostraws were transported (with dry ice) to our laboratory in Berkeley, CA, where they were maintained at -80°C . After opening each cryostraw, 20 μL of serum was mixed with 100 μL of a solvent mixture (isopropanol/methanol/water = 60:35:5) containing ^{13}C -cholic acid as an internal standard (3.0 $\mu\text{g}/\text{mL}$). After mixing samples for one minute with a vortex mixer, samples were allowed to stand at room temperature for 10 minutes to precipitate proteins and were then centrifuged for 10 minutes at $10,000 \times g$. The supernatant was removed and stored at 4°C prior to liquid chromatography (LC)-HRMS. Case-control pairs were analyzed sequentially but in random order. A local quality control sample, prepared by pooling aliquots from each serum sample, was analyzed as each tenth injection to provide technical replicates for estimating precision.

LC-HRMS was performed on two platforms. The first 132 samples were analyzed with an Agilent LC (1100 series) coupled to an Agilent HRMS (Model 6550 QTOF). Because of a malfunction, this QTOF required repairs before analyses could be completed. To permit timely analysis, the remaining 58 samples were analyzed with an Agilent 1200 series LC coupled to an LTQ Orbitrap XL HRMS equipped with an Ion Max ESI source (Thermo Fisher Scientific). On both platforms, 10 μL of each sample was injected from a full loop into a Luna C5 column (2.1×50 mm, 100 \AA , 5 μm , Phenomenex) operated with gradient elution of mobile phase A (methanol/0.5% acetic acid = 5:95) and mobile phase B (isopropanol/methanol/0.5% acetic acid = 60:35:5) as follows: 100% A for 2 minutes at 0.05 mL/minutes; 0–83% B from 2 to 7 minutes at 0.3 mL/minute; 83–100% B from 7 to 14 minutes at 0.3 mL/minute; 100% B from 14 to 17 minutes; and 100% A from 17 to 22 minutes. The autosampler and column oven were maintained at 4°C and 40°C , respectively. The electrospray was operated in negative ionization mode. To monitor system stability, pooled quality control samples were injected every tenth sample. Tandem MS/MS spectra were obtained with the Orbitrap platform.

During processing, approximately one third of the serum samples was observed to have a gelled consistency that apparently resulted from a preservative(s) contained in the cryostraws (25, 26); gelled serum from EPIC cryostraws has been observed previously (27). Pairs with at least one gelled sample were analyzed in a single batch (batch 1, $n = 96$) on the QTOF platform, and the remaining (non-gelled) pairs were analyzed in two batches on either the QTOF platform (batch 2, $n = 36$) or the Orbitrap platform (batch 3, $n = 58$).

Several fresh seafood samples were purchased from a local market in Berkeley, CA and tested for the presence of ULCEAS. Four types of seafood were tested: raw white shrimp (Thailand), wild American sea scallops, and farmed American Littleneck clams and live mussels. Samples from these four species (50 μL) were extracted for lipids using the Bligh and Dyer chloroform extraction method (28, 29). These extracts were analyzed on the Orbitrap platform, with the same method as described above.

Data processing

Raw data were converted to MZXML format for peak picking using ProteoWizard software (Spielberg Family Center for Applied Proteomics, Los Angeles, CA). Peak detection and retention time alignment were performed with the XCMS package within the R statistical programming environment (30, 31). For the data collected on the QTOF, parameters include centwave

Table 1. Descriptive statistics of human subjects from the EPIC cohort matched by age, study enrollment year and season, and gender

Total $n = 190$	Colorectal cancer cases, $n = 95$	Controls, $n = 95$	<i>P</i>
Gender			
Male	68	68	
Female	27	27	
Age at enrollment, y			
Median	57	57	
Min	36	35	
Max	65	64	
Years to diagnosis (from enrollment)			
Median	7.1	—	
Min	0.1	—	
Max	14.4	—	
BMI			
Median	26.4	25.1	0.0090
Min	19.6	18.7	
Max	40.6	33.6	
Waist circumference (cm)			
Median	95	90	0.0005
Min	68	64	
Max	115	119	
Dietary fish (g/d)			
Median	21	24	0.1660
Min	1	0	
Max	77	83	
Dietary shellfish (g/d)			
Median	4	3	0.4526
Min	0	0	
Max	45	76	

Table 2. ULCFAs reported by Ritchie and colleagues (11) detected in the current investigation

ULCFA	Formula	Theoretical m/z^a	Observed m/z^a	Mass dev. (ppm)	Ret. time (sec)	Peak shape ^b	CV
446	C ₂₈ H ₄₆ O ₄	445.3327	445.3324	0.70	610.94	Pass	0.276
448	C ₂₈ H ₄₈ O ₄	447.3483	447.3470	3.01	615.20	Pass	0.262
466	C ₂₈ H ₅₀ O ₅	465.3590	465.3586	0.88	583.05	Pass	0.276
468	C ₂₈ H ₅₂ O ₅	467.3742	467.3744	-0.38	605.56	Pass	0.181
492	C ₃₀ H ₅₂ O ₅	491.3741	491.3735	1.22	612.33	Pass	0.185
494	C ₃₀ H ₅₄ O ₅	493.3896	493.3906	-1.96	612.28	Pass	0.236
518	C ₃₂ H ₅₄ O ₅	517.3902	517.3883	3.59	616.13	Fail	ND
538	C ₃₂ H ₅₈ O ₆	537.4164	537.4155	1.58	604.36	Pass	0.091
574	C ₃₆ H ₆₂ O ₅	573.4527	573.4508	3.33	611.53	Fail	ND
576	C ₃₆ H ₆₄ O ₅	575.4683	575.4666	2.97	616.40	Fail	ND
578	C ₃₆ H ₆₆ O ₅	577.4837	577.4842	-0.79	629.90	Fail	ND
592	C ₃₆ H ₆₄ O ₆	591.4630	591.4637	-1.21	613.37	Fail	ND
594	C ₃₆ H ₆₆ O ₆	593.4786	593.4783	0.42	616.41	Pass	0.252

Abbreviations: CV, coefficient of variation; m/z , mass-to-charge ratio; ND, not determined.

^aTheoretical and observed m/z values correspond to singly charged negative ions.

^bBased upon visual inspection of peak morphology for all selected-ion chromatograms.

feature detection, orbiswarp retention time correction, minimum fraction of samples in one group to be a valid group = 0.25, P value thresholds for blank versus QC samples = 0.01, isotopic ppm error = 10, width of overlapping m/z slices ($mzwid$) = 0.015, bandwidth grouping (bw) = 2, minimum peak width = 2 s, maximum peak width = 20 s. Parameters for the Orbitrap platform were the same except for: isotopic ppm error = 2.5, minimum peak width = 2 s, maximum peak width = 70 s, bw = 5, prefilter peaks = 3, prefilter intensity = 5000, based on XCMS parameters optimized for Orbitrap instruments (32). The resulting peak tables of retention times, m/z values, and peak intensities were exported for further processing. Subsequent analyses were also performed with the R platform (version 3.2.1; ref. 33).

Because reference standards for the ULCFAs are not available, mass spectra were interrogated for 13 accurate masses representing ULCFAs with between 28 and 36 carbons that had been reported by Ritchie and colleagues (11, 17). These ULCFAs are listed in Table 2 along with their masses and elemental formulae. We targeted these 13 ions in our analyses and Table 2 shows the retention times and observed masses, along with the mass accuracy expressed as the mass deviation (ppm) between the theoretical and observed masses. Tandem MS analyses revealed fragment ions representing losses of CO₂ and one or two H₂O molecules for all 13 precursor ions. These losses are consistent with hydroxylated carboxylic acids and with fragment ions reported by Ritchie and colleagues (11). After extracting accurate masses for the 13 putative ULCFAs from total-ion chromatograms for all EPIC specimens, extracted-ion chromatograms were visually examined and five of the features were excluded because some peaks were not reproducibly detected above noise levels (ULCFAs 518, 574, 576, 578, and 592; Table 2).

For quantitation of ULCFA levels, we followed the same approach as Ritchie and colleagues (12) and normalized analyte peak areas by the corresponding peak areas of an internal standard (¹³C-cholic acid, final concentration = 3.0 μg/mL). These normalized ULCFA abundances are designated as "peak-area ratios" (PAR). Preliminary statistical analyses indicated that use of PARs, rather than simply ULCFA peak areas, reduced nuisance variation from instrumental variability and matrix effects.

Statistical analysis

Batch adjustment was performed with a linear model of the log-transformed PAR of each analyte, which included dummy variables for batch and gel status as independent variables. Residuals from these linear models were used as dependent variables in subsequent statistical analyses. These residuals represent log-transformed PAR values normalized to a mean of zero. Coefficients of variation (CV) for the eight ULCFAs with acceptable peak morphology were estimated from the error variances (σ_e^2) of log-transformed PARs after batch and gel adjustment as $\sqrt{e^{\sigma_e^2} - 1}$ (ref. 34; Table 2).

Analyte levels were compared between cases and controls using one-sided paired t tests as well as the linear model (1) for evaluating both case-control differences and effects of TTD (Table 3). Additional linear models were constructed by adding BMI, waist circumference, and self-reported consumption of fish and shellfish to model (1) as covariates (Table 4). Waist circumference had previously been associated with colorectal cancer (23, 24) and consumption of fish and shellfish introduces n-3 fatty acids into the diet that purportedly reduce cancer risks (18, 19) and are metabolized to anti-inflammatory lipoxins, resolvins, and protectins (14). As noted above, some serum

Table 3. Difference in means from one-sided t tests of cases and controls and coefficients from linear model (1) which regressed, case-control differences on TTD

ULCFA	Paired t test		Linear model (1)				
	t -statistic	P	β_0	P	$\beta_1 (\times 10^3)$	P	R^2
446	-0.237	0.0116	-0.626	0.0037	0.150	0.0373	0.046
448	-0.139	0.0581	-0.390	0.0342	0.097	0.1186	0.026
466	-0.203	0.0139	-0.633	0.0008	0.166	0.0086	0.072
468	-0.215	0.0064	-0.567	0.0014	0.136	0.0219	0.055
492	-0.126	0.0873	-0.490	0.0104	0.140	0.0291	0.050
494	-0.183	0.0300	-0.536	0.0076	0.136	0.0430	0.043
538	-0.108	0.1193	-0.367	0.0527	0.100	0.1169	0.026
594	-0.008	0.4700	-0.238	0.2741	0.089	0.2281	0.016

NOTE: β_0 is the model intercept representing the case-control difference at recruitment and β_1 is the regression coefficient for TTD.

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Table 4. Results of adding covariates to linear model (1), where the dependent variable is the difference between logged levels of the particular ULCFA in a case-control pair

ULCFA	BMI <i>P</i>	ΔR^2	Waist circumference <i>P</i>	ΔR^2	Dietary fish <i>P</i>	ΔR^2	Dietary shellfish <i>P</i>	ΔR^2
446	0.4114	0.008	0.1402	0.012	0.5714	-0.013	0.3225	-0.005
448	0.8771	0.001	0.5706	-0.001	0.2390	0.017	0.7647	0.001
466	0.1915	0.018	0.3259	-0.016	0.6431	0.012	0.1849	0.030
468	0.1092	0.025	0.7061	-0.007	0.9843	0.016	0.3709	0.026
492	0.1002	0.021	0.3488	0.016	0.6982	0.031	0.7683	0.030
494	0.1664	0.018	0.5955	-0.012	0.5069	0.018	0.5631	0.017
538	0.0199	0.057	0.2055	0.016	0.2654	0.030	0.9275	0.015
594	0.0259	0.052	0.1947	0.019	0.1316	0.050	0.9861	0.023

NOTE: BMI is the body mass index.

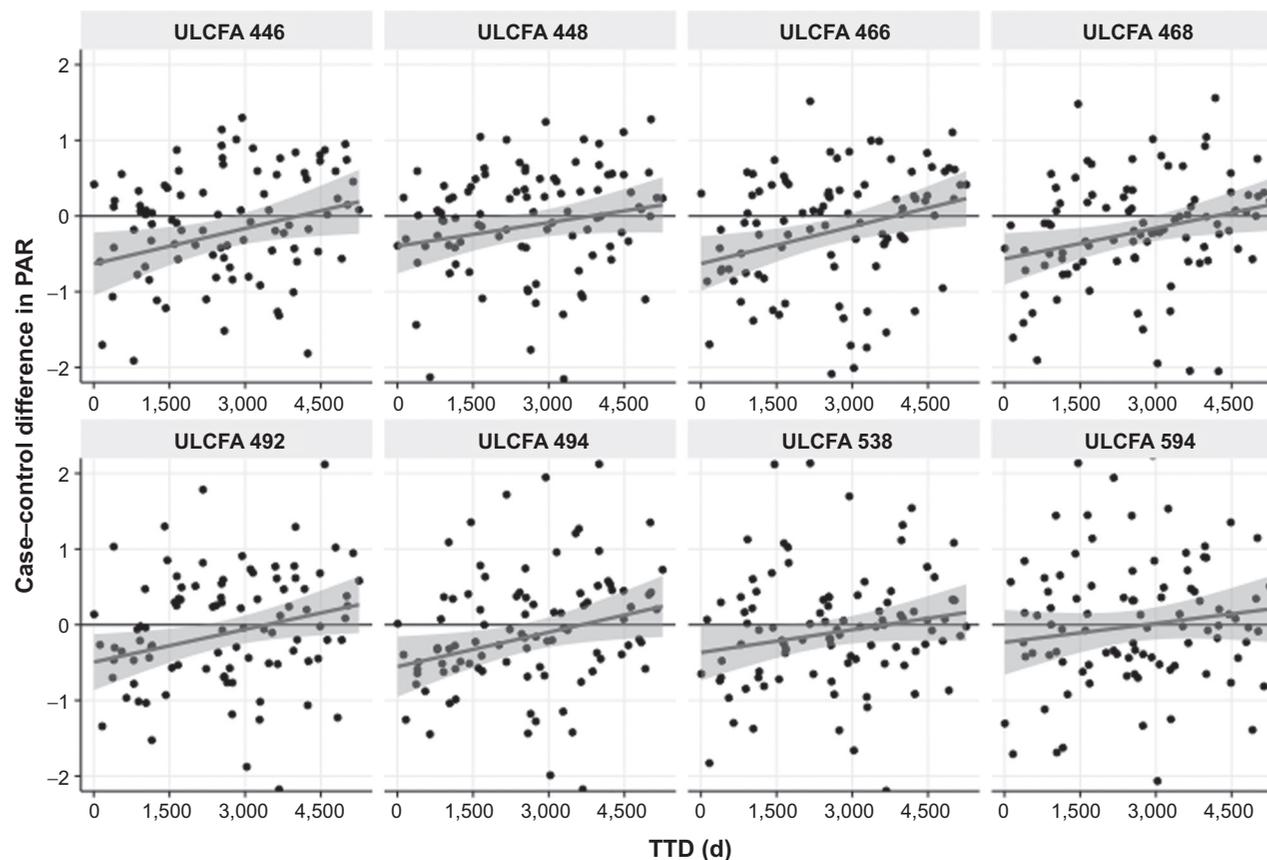
samples had a gelled consistency. When gel status was added to linear models, no significant main effect or interaction between case-control status and gel status was detected (results not shown).

Results

Approximately normal distributions of logged ULCFA PARs were verified for all three batches, and Kruskal-Wallis tests detected no significant differences across batches ($P > 0.33$). As

indicated in Table 2, CVs ranged from 9.1 to 27.6% (mean 22%) for the 8 ULCFAs with acceptable peak morphology.

As shown in Table 3, paired *t* tests detected significantly lower PARs in cases compared with controls for four 28-carbon ULCFAs (446, 466, 468, and 494). Significant case-control differences of PARs were confirmed with a negative intercept from model (1) for the same 28-carbon ULCFAs and a fifth 30-carbon ULCFA (492). Interestingly, these five ULCFAs also showed statistically significant coefficients for TTD. Indeed, as shown in Fig. 2, PAR differences between cases and controls decreased with increasing

**Figure 2.**

Linear-model plots. Case-control differences for ULCFA levels versus time to diagnosis [TTD; model (1)]. Each point represents the difference in log-transformed peak-area ratios (PAR; ULCFA/¹³C-choleic acid), normalized to a mean of zero, for a given case-control pair after batch adjustment. Error bands represent 95% confidence intervals.

TTD for all 8 ULCFAs. Since case–control differences in levels of these ULCFAs appear to decline with increasing TTD, we conclude that these molecules are reactive biomarkers of colorectal cancer progression rather than biomarkers of protective exposure, as hypothesized by Ritchie and colleagues (12).

Table 4 shows results from extensions of model (1) to include BMI, waist circumference, and self-reported consumption of fish and shellfish. As the matched pairs were also matched on gender, the relationship between ULCFAs and gender was tested with an unpaired *t* test and no significant difference was observed. The only significant associations observed between these covariates and case–control differences in PAR values were those for ULCFAs 538 and 594 with increasing BMI. No ULCFA peaks were distinguishable from background noise in the seafood samples.

Although our study confirms that levels of ULCFAs with 28–30 carbons are significantly lower in incident colorectal cancer cases than matched controls (11), the influence of TTD on case–control differences (Fig. 2) suggests that these fatty acids are more likely to be markers of colorectal cancer progression rather than biomarkers of protective exposure.

Evidence that lower levels of ULCFAs may be linked to the progression of colorectal cancer points to tumor-induced metabolism as a likely contributor, but leaves open the question as to the origins of the molecules. Although Ritchie and colleagues readily observed ULCFAs in human serum, they failed to detect the same molecules in sera from rats, mice, and cattle, in various plant tissues and grains, and in human cell lines from tumors and normal colonic tissue (11). Aside from carbon chain length, the proposed structures of ULCFAs (35) resemble those of the lipoxins, resolvins, and protectins (20–22 carbons); these are mono-, di-, and tri-hydroxylated products of long-chain fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that have been decarboxylated through metabolism (36–39). As EPA and DHA are present in oily tissues from marine species, we suspected that the ULCFAs might also be present in seafood. However, we did not detect ULCFAs in commercial samples of shrimp, scallops, clams, or mussels.

While the origin of hydroxylated ULCFAs remains unknown, very-long chain (VLC) PUFAs, ranging from 22–34 carbons, have been described (40, 41) and detected in spermatozoa, retinas, and brain tissue (42, 43). PUFAs longer than 22 carbons are generated by elongase ELOVL-4, which is one of seven endoplasmic reticulum-bound enzymes responsible for lengthening particular fatty acids (44). While these VLC-PUFAs are not typically hydroxylated, it is plausible that they share common synthetic pathways with the hydroxylated ULCFAs described by Ritchie and colleagues. Alternatively, elongases ELOVL2 and ELOV5 extend typical-length PUFAs (18–22 carbon) but have not been investigated as possible progenitors of ULCFAs (45).

Our approach for simultaneously comparing paired case–control differences as a function of TTD, embodied in model (1), offers an efficient mechanism for differentiating biomarkers of exposure from those of disease progression and is sufficiently general for use with either targeted or untargeted analyses of biospecimens from prospective cohorts. Previous analyses that employed TTD in studies of disease etiology have been restricted to biomarker levels in cases only (22, 46, 47) and have also been used to exclude cases diagnosed relatively soon after specimen collection (e.g., 2–5 years) (48–50).

For the colorectal cancer case–control samples evaluated in the current study, the 28-carbon ULCFAs were the class most highly

associated with case status and TTD (Table 3). Ritchie and colleagues reported that several 36-carbon compounds were also highly discriminating between cases and controls for both colorectal cancer (11, 13) and pancreatic cancer (17, 51). However, the only 36-carbon ULCFA that we were able to quantify was 594, which was not significantly associated with either colorectal cancer case status or TTD (Table 3), although the plot in Fig. 2 suggests a weak, but consistent, trend with TTD.

Discussion

Although our results tend to downplay the potential roles of ULCFAs as biomarkers of protective exposure, they may be worth evaluating as diagnostic biomarkers of colorectal cancer. Indeed, relationships shown in Table 3 point to significant reductions in three of the 28-carbon ULCFAs (446, 466, and 468) starting between about 1,500–3,000 days (3–7 years) prior to diagnosis.

We emphasize that our methods relied on accurate masses to pinpoint ULCFAs and employed quantitation relative to ¹³C-cholic acid (internal standard). With availability of reference standards, it would be possible to detect and quantitate these molecules with greater precision and thus to reduce measurement errors and resulting attenuation biases that probably weakened associations observed with colorectal cancer status and TTD. However, improved standardization would be unlikely to remove the consistent effects of TTD that were observed in our samples of colorectal cancer cases and controls from the EPIC cohort (Fig. 2).

We recognize that our study is small and has limited power to detect associations between ULCFAs and colorectal cancer. Nonetheless, these results offer important clues that the ULCFAs might be useful diagnostic markers. Validation with larger sample sets is now necessary.

In conclusion, these targeted analyses of 8 accurate masses, which are characteristic of ULCFAs reported by Ritchie and colleagues in case–control studies (11), confirmed that some ULCFAs were present at significantly lower levels in incident colorectal cancer cases than matched controls from the EPIC cohort. However, clear trends with TTD indicate that the observed case–control differences are unlikely to be due to the ULCFAs acting as protective exposures but rather reflect progression of the disease. Although ULCFAs are probably not involved with causal pathways leading to colorectal cancer, their correlations with TTD suggest that they may be useful diagnostic biomarkers. Future research regarding applications of these molecules in cancer research would benefit from synthesis of reference standards and knowledge of the dietary or metabolic origins of these novel molecules.

Our use of a linear model that employed TTD as a covariate [model (1)] provides an efficient method for distinguishing causal and reactive biomarkers in specimens of blood from prospective cohorts. The model is simple to apply and is sufficiently general for use with either targeted or untargeted analyses of biospecimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: K. Perttula, W.M.B. Edmands, H. Grigoryan, X. Cai, S.M. Rappaport

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References

- Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. *CA Cancer J Clin* 2014;64:104–17.
- Howlander N, Noone A, Krapcho M, Garshell J, Miller D, Altekruse S, et al. SEER Cancer Statistics Review, 1975–2011 [Internet]. 2014. Available from: http://seer.cancer.gov/csr/1975_2011/.
- Hemminki K, Czene K. Attributable risks of familial cancer from the family-cancer database. *Cancer Epidemiol Biomarkers Prev* 2002;64:1638–44.
- Rappaport SM. Genetic factors are not the major causes of chronic diseases. *PLoS One* 2016;11:e0154387.
- Stone WL, Krishnan K, Campbell SE, Palau VE. The role of antioxidants and pro-oxidants in colon cancer. *World J Gastrointest Oncol* 2014;6:55–66.
- Rothwell PM, Fowkes FGR, Belch JFF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2010;377:31–41.
- Awtry E, Loscalzo J. Aspirin. *Circulation*. 2000;101:1206–18.
- Chiang N, Arita M, Serhan CN. Anti-inflammatory circuitry: lipoxin, aspirin-triggered lipoxins and their receptor ALX. *Prostaglandins Leukot Essent Fatty Acids* 2005;73:163–77.
- Terzić J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. *Gastroenterology* 2010;138:2101–14.
- Masoodi M, Mir AA, Petasis NA, Serhan CN, Nicolaou A. Simultaneous lipidomic analysis of three families of bioactive lipid mediators leukotrienes, resolvins, protectins and related hydroxy-fatty acids by liquid chromatography/electrospray tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2008;22:75–83.
- Ritchie SA, Ahiahonu PWK, Jayasinghe D, Heath D, Liu J, Lu Y, et al. Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids in the serum of colorectal cancer patients: implications for early screening and detection. *BMC Med* 2010;8:13.
- Ritchie SA, Heath D, Yamazaki Y, Grimmalt B, Kavianpour A, Krenitsky K, et al. Reduction of novel circulating long-chain fatty acids in colorectal cancer patients is independent of tumor burden and correlates with age. *BMC Gastroenterol* 2010;10:140.
- Ritchie SA, Tonita J, Alvi R, Lehotay D, Elshoni H, Su-Myat, et al. Low-serum GTA-446 anti-inflammatory fatty acid levels as a new risk factor for colon cancer. *Int J Cancer* 2013;132:355–62.
- Ritchie SA, Jayasinghe D, Davies GF, Ahiahonu P, Ma H, Goodenowe DB. Human serum-derived hydroxy long-chain fatty acids exhibit anti-inflammatory and anti-proliferative activity. *J Exp Clin Cancer Res* 2011;30:59.
- Babbs CF. Free radicals and the etiology of colon cancer. *Free Radic Biol Med* 1990;8:191–200.
- Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010;376:1741–50.
- Ritchie SA, Akita H, Takemasa I, Eguchi H, Pastural E, Nagano H, et al. Metabolic system alterations in pancreatic cancer patient serum: potential for early detection. *BMC Cancer* 2013;13:416.
- Larsson SC, Kumlin M, Ingelman-sundberg M, Wolk A. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms 1–3. *Am J Clin Nutr* 2004;79:935–45.
- Song M, Chan AT, Fuchs CS, Ogino S, Hu FB, Mozaffarian D, et al. Dietary intake of fish, ω -3 and ω -6 fatty acids and risk of colorectal cancer: a prospective study in U.S. men and women. *Int J Cancer* 2014;135:2413–23.
- Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. *Public Health Nutr* 2002;5:1113–24.
- Williams MD, Reeves R, Resar LS, Hill HH. Metabolomics of colorectal cancer: past and current analytical platforms. *Anal Bioanal Chem* 2013;405:5013–30.
- Nolen BM, Brand RE, Prosser D, Velikokhatnaya L, Allen PJ, Zeh HJ, et al. Prediagnostic serum biomarkers as early detection tools for pancreatic cancer in a large prospective cohort study. *PLoS One* 2014;9:e94928.
- Moore LL, Bradlee ML, Singer MR, Splansky GL, Proctor MH, Ellison RC, et al. BMI and waist circumference as predictors of lifetime colon cancer risk in Framingham Study adults. *Int J Obes Relat Metab Disord* 2004;28:559–67.
- Aleksandrova K, Boeing H, Jenab M, Bueno-de-Mesquita HB, Jansen E, Van Duynhoven FJB, et al. Metabolic syndrome and risks of colon and rectal cancer: the European prospective investigation into cancer and nutrition study. *Cancer Prev Res* 2011;4:1873–83.
- Talwar P. Manual of assisted reproductive technologies and clinical embryology. New Delhi, India: Jaypee Brothers Medical Publisher Pvt. Limited; 2014.
- Saint-Ramon J-G, Beau C, Ehrsam A, inventors; Imv Technologies, assignee. Tubes for conservation biological particle; for use as tool in biological sampling. Patent number: US6300125 B1. Google Patents; 2001 Oct. 9.
- Fages A, Ferrari P, Monni S, Dossus L, Floegel A, Mode N, et al. Investigating sources of variability in metabolomic data in the EPIC study: the Principal Component Partial R-square (PC-PR2) method. *Metabolomics* 2014;10:1074–83.
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–7.
- Schlechtriem C, Focken U, Becker K. Effect of different lipid extraction methods on delta13C of lipid and lipid-free fractions of fish and different fish feeds. *Isotopes Environ Health Stud* 2003;39:135–40.
- Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. LC/MS preprocessing and analysis with XCMS. *Anal Chem* 2006;78:779–87.
- Benton HP, Wong DM, Trauger SA, Siuzdak G. XCMS 2: processing tandem mass spectrometry data for metabolite identification and structural characterization. *Anal Chem* 2008;80:6382–9.
- Patti GJ, Tautenhahn R, Siuzdak G. Meta-analysis of untargeted metabolomic data from multiple profiling experiments. *Nat Protoc* 2012;7:508–16.
- R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013. Available from: <http://www.R-project.org/>.
- Aitchison J, Brown JA. The lognormal distribution. London, England: Cambridge University Press; 1957.
- Ritchie S, Goodenowe D, Khan MA, Ahiahonu PWK, inventors; Phenomenon Discoveries Inc., assignee. Hydroxy fatty acid compounds and uses thereof for disease treatment and diagnosis. Patent WO2011011882 A1. 2011 Feb 3.
- Serhan CN. Novel eicosanoid and docosanoid mediators: resolvins, docosatrienes, and neuroprotectins. *Curr Opin Clin Nutr Metab Care* 2005;8:115–21.

37. Schwab JM, Serhan CN. Lipoxins and new lipid mediators in the resolution of inflammation. *Curr Opin Pharmacol* 2006;6:414–20.
38. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 2008;8:349–61.
39. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 2014;510:92–101.
40. Aveldano MI, Robinson BS, Johnson DW, Poulos A. Long and very long chain polyunsaturated fatty acids of the n-6 series in rat seminiferous tubules. *J Biol Chem* 1993;268:11663–9.
41. Robinson BS, Johnson DW, Poulos A. Novel molecular species of sphingomyelin containing 2-hydroxylated polyenoic very-long-chain fatty acids in mammalian testes and spermatozoa. *J Biol Chem* 1992; 267:1746–51.
42. Poulos A. Very long chain fatty acids in higher animals—a review. *Lipids* 1995;30:1–14.
43. Agbaga M-P, Mandal MN, Anderson RE. Retinal very long-chain PUFAs: new insights from studies on ELOVL4 protein. *J Lipid Res* 2010;51: 1624–42.
44. Leonard AE, Pereira SL, Sprecher H, Huang YS. Elongation of long-chain fatty acids. *Prog Lipid Res* 2004;43:36–54.
45. Jakobsson A, Westerberg R, Jakobsson A. Fatty acid elongases in mammals: their regulation and roles in metabolism. *Prog Lipid Res* 2006;45: 237–49.
46. Vickers AJ, Ullmert D, Serio AM, Björk T, Scardino PT, Eastham JA, et al. The predictive value of prostate cancer biomarkers depends on age and time to diagnosis: towards a biologically-based screening strategy. *Int J Cancer* 2007;121:2212–7.
47. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. *JAMA* 2004;291:585–90.
48. Dorgan JF, Longcope C, Stephenson HE, Falk RT, Miller R, Franz C, et al. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1996;5:533–9.
49. McSorley MA, Alberg AJ, Allen DS, Allen NE, Brinton LA, Dorgan JF, et al. C-reactive protein concentrations and subsequent ovarian cancer risk. *Obstet Gynecol* 2007;109:933–41.
50. Cust AE, Kaaks R, Friedenreich C, Bonnet F, Laville M, Lukanova A, et al. Plasma adiponectin levels and endometrial cancer risk in pre- and postmenopausal women. *J Clin Endocrinol Metab* 2007;92:255–63.
51. Ritchie SA, Chitou B, Zheng Q, Jayasinghe D, Jin W, Mochizuki A, et al. Pancreatic cancer serum biomarker PC-594: diagnostic performance and comparison to CA19-9. *World J Gastroenterol* 2015;21:6604–12.

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