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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 (ULCFAs) that were present at significantly lower levels in colorectal cancer (CRC) cases than controls. Follow-up studies suggested that these molecules were potential biomarkers of protective exposure for CRC. To test the hypothesis that ULCFAs reflect causal pathways, we measured their levels in prediagnostic serum from incident CRC cases and controls.

Methods: Serum from 95 CRC 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 CRC cases, confirming earlier reports, the case-control differences diminished significantly with increasing *ttd* (7 d to 14 y).

Conclusion: Levels of several ULCFAs were lower in incident CRC 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.

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Impact: ULCFA levels are unlikely to represent exposures that protect individuals from CRC. 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.

Keywords

colorectal cancer; causal biomarkers; reactive biomarkers; ultra-long-chain fatty acids; EPIC

Introduction

Colorectal cancer (CRC) accounts for one fourth of all cancer deaths worldwide and is the second leading cause of cancer mortality in the United States and Europe (1,2). Since less than 15 percent of the variation in risk of CRC 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 CRC development. Exposures that have been associated with increased risks of CRC 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, *et al.* (5)). On the other hand, regular consumption of aspirin – an antioxidant and anti-inflammatory drug - reduces CRC 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) (8). This combination of factors suggests that CRC 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 *et al.*, used untargeted high-resolution mass spectrometry (HRMS) to detect a novel class of polyunsaturated, hydroxylated, ultra-long-chain fatty acids (ULCFAs, containing between 28 and 36 carbons) that was associated with reduced risks of CRC in three case-control studies (11). Using accurate-mass signatures of a dozen representative ULFCAs, Ritchie *et al.* 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 CRC 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 CRC risk of 10.1 (C.I.: 6.4 – 16.4) (13).

In attempting to elucidate a protective mechanism for these molecules, Ritchie *et al.* dosed human CRC (SW620) cells with 28-carbon ULFCAs that had been isolated from human serum, and reported reduced production of pro-inflammatory markers (NFκB, IκBα, and NOS2) (14). Since, as noted above, inflammation has been a hallmark of CRC (5,9,15), the inverse correlation of ULCFA levels and CRC risk would be consistent with a cancer mechanism that favors a pro-inflammatory environment that increases with age.

Furthermore, the purported anti-inflammatory or protective properties of ULCFAs 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 pM – nM range (10) and have epimeric forms that are triggered by aspirin, which reduces risks of CRC and cancer generally (6,16).

Remarkably, the provocative findings of Ritchie *et al.* (11–14,17) implicating low serum levels of ULCFAs as potential causes of CRC have not been explored by other investigators. Since all of the reported associations between circulating levels of ULFCAs and CRC 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 CRC diagnosis. This would reduce the likelihood that lower levels of ULFCAs in CRC cases resulted from tumor-induced dysregulation of homeostatic pathways (reverse causality). The purpose of this study is to test the hypothesis that ULFCAs are potentially protective against CRC with pre-diagnostic serum from 95 incident CRC cases and matched controls from the European Prospective Investigation of Cancer and Nutrition (EPIC). Also, since previous reports had implicated consumption of seafood as being potentially protective of CRC (18,19), several fresh seafood samples were tested for the presence of ULCFAs.

Materials and Methods

Experimental Design

We adopted a simple regression model to determine whether ULCFAs represent biomarkers on the causal pathway to CRC or are reactive biomarkers related to progression of the disease. Since the EPIC serum had been obtained between 7 d and 14 y prior to CRC diagnosis, we used the (log-scale) difference in ULFCA concentrations (CRC 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 CRC. (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) ULCFA 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 ULCFA level differed on average between cases and controls. A negative intercept, illustrated with the hypothetical example in Figure 1A, would indicate higher ULCFA levels in controls (*i.e.* a protective effect) as suggested by Ritchie *et al.* (11). Likewise, a significant coefficient for *ttd* (β_1), illustrated in Figure 1B, would indicate that the timing of blood collection relative to diagnosis affected the outcome and, therefore, that any case-control difference in the ULFCA level probably reflects progression of CRC. Thus, the combination of a negative β_0 and non-significant β_1 would point to a potentially causal biomarker of CRC 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, aged 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 based on previous evidence that BMI and waist circumference are associated with CRC 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 (Milwaukee, WI, USA). LC-MS grade (Optima) acetic acid and chloroform were from Fisher Scientific (Santa Clara, CA, USA). All chemicals were of analytical 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 (USA), 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 μg/ml). After mixing samples for one minute with a vortex mixer, samples were allowed to stand at room temperature for 10 min. to precipitate proteins and were then centrifuged for 10 min at 10,000 *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.

Liquid chromatography-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, Santa Clara, CA, USA). Due to a malfunction, this QTOF required repairs before analyses could be completed. In order to permit timely analysis, the remaining 58 samples were analyzed with an Agilent 1200 series LC (Santa Clara, CA, USA) coupled to an LTQ Orbitrap XL HRMS equipped with an Ion Max ESI source (Thermo Fisher Scientific, Waltham, MA, USA). On both platforms, 10 μl of each sample was injected from a full loop into a Luna C5 column (2.1 × 50 mm, 100 Å, 5 μm, Phenomenex, Los Angeles, CA) 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/min; 0–83% B from 2–7 minutes at 0.3 ml/min; 83–100% B from 7–14 minutes at 0.3 ml/min; 100% B from 14–17 minutes; and 100% A from 17–22 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, California and tested for the presence of ULCFAs. 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 feature detection, orbiwarp 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) (33).

Because reference standards for the ULCFAs are not available, mass spectra were interrogated for 13 accurate masses representing ULFCAs with between 28 and 36 carbons that had been reported by Ritchie *et al* (11,17). These ULFCAs 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 CO_2 and one or two H_2O molecules for all 13 precursor ions. These losses are consistent with hydroxylated carboxylic acids and with fragment ions reported by Ritchie, *et al.* (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 (ULFCAs 518, 574, 576, 578, and 592) (Table 2).

For quantitation of ULCFA levels, we followed the same approach as Ritchie *et al.* (12) and normalized analyte peak areas by the corresponding peak areas of an internal standard (^{13}C-cholic acid, final concentration = 3.0 μg/ml). These normalized ULCFA abundances are designated as 'peak-area ratios' (PARs). 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 (CVs) 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 $\sqrt{e^{\sigma_e^2} - 1}$ (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 CRC (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 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*-value > 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 to 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 time to diagnosis (*ttd*). Indeed, as shown in Figure 2, PAR differences between cases and controls decreased with increasing *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 CRC progression rather than biomarkers of protective exposure, as hypothesized by Ritchie, *et al.* (12).

Table 4 shows results from extensions of model (1) to include BMI, waist circumference, and self-reported consumption of fish and shellfish. Since 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 CRC cases than matched controls (11), the influence of *ttd* on case-control differences (Figure 2) suggests that these fatty acids are more likely to be markers of CRC progression rather than biomarkers of protective exposure.

Evidence that lower levels of ULFCAs may be linked to the progression of CRC points to tumor-induced metabolism as a likely contributor, but leaves open the question as to the origins of the molecules. Although Ritchie *et al.* 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). Since 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, *et al.* 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 CRC 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, *et al.* reported that several 36-carbon compounds were also highly discriminating between cases and controls for both CRC (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 CRC case status or *ttd* (Table 3), although the plot in Figure 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 CRC. Indeed, relationships shown in Table 3 point to significant reductions in three of the 28-carbon ULCFAs (446, 466, & 468) starting between about 1,500 – 3,000 d (3 – 7 y) prior to diagnosis.

We emphasize that our methods relied on accurate masses to pinpoint ULCFAs and employed quantitation relative to ^{13}C-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 CRC status and *ttd*. However, improved standardization would be unlikely to remove the consistent effects of *ttd* that were observed in our samples of CRC cases and controls from the EPIC cohort (Figure 2).

We recognize that our study is small and has limited power to detect associations between ULCFAs and CRC. 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 *et al.* in case-control studies (11), confirmed that some ULCFAs were present at significantly lower levels in incident CRC 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 CRC, 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.

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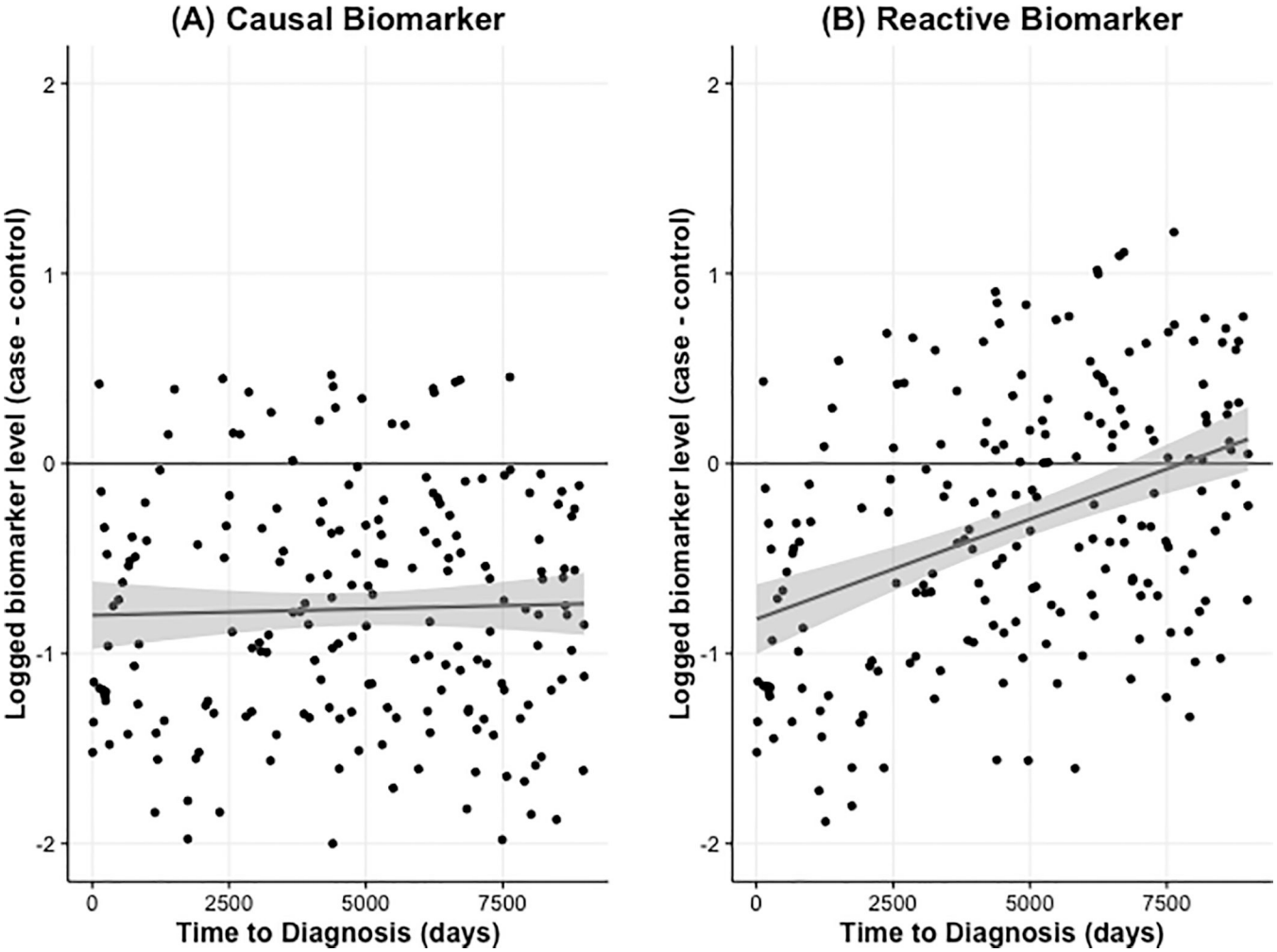

Figure 1. Use of a linear model (model 1) to differentiate a causal biomarker from a disease-related biomarker.

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.

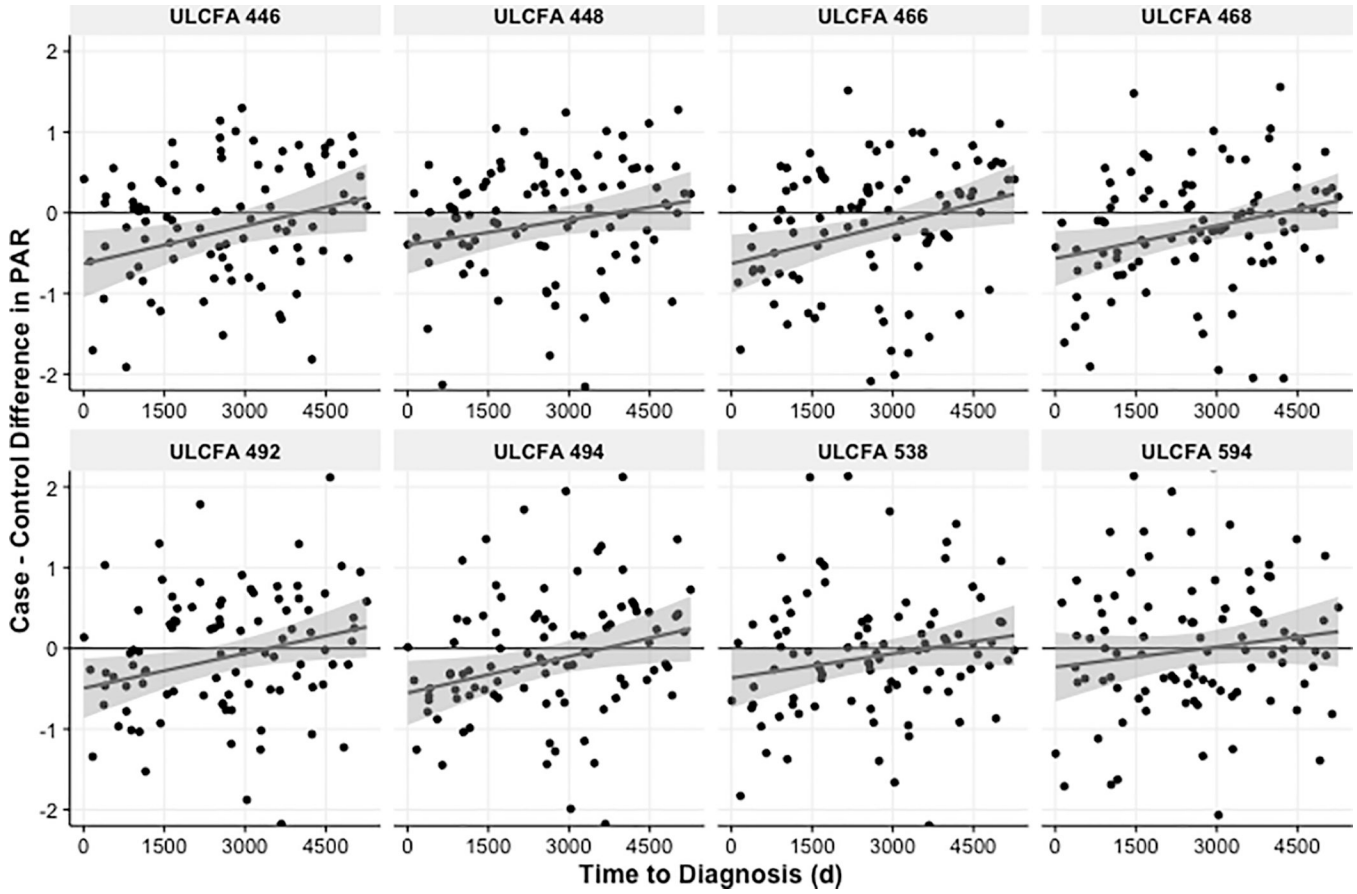

Figure 2. Linear-model plots.

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

Table 1.

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

		Total *n*=190	CRC cases *n*=95	Controls *n*=95	*P*-value
Gender	Male	68	68		
	Female	27	27		
Age at enrollment	median	57	57		
(y)	min	36	35		
	max	65	64		
Years to diagnosis	median	7.1	-		
(from enrollment)	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	median	95	90	0.0005	
(cm)	min	68	64		
	max	115	119		
Dietary fish	median	21	24	0.1660	
(g/d)	min	1	0		
	max	77	83		
Dietary shellfish	median	4	3	0.4526	
(g/d)	min	0	0		
	max	45	76		

Table 2.

Ultra-long-chain fatty acids (ULCFAs) reported by Ritchie, *et al.* (8) 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

Legend: *m/z* is the mass-to-charge ratio; CV is the coefficient of variation; ND indicates not determined.

[a] Theoretical and observed *m/z* values correspond to singly-charged negative ions.

[b] Based upon visual inspection of peak morphology for all selected-ion chromatograms.

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 time to diagnosis (*ttd*).

ULCFA	Paired t-test		Linear model (1)				
	t-Stat.	*P*-value	β_0	*P*-value	β_1 ($\times 10^3$)	*P*-value	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

Legend : β_0 is the model intercept representing the case-control difference at recruitment and β_1 is the regression coefficient for *ttd*.

Table 4:

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

ULCFA	BMI *P*-value	ΔR^2	Waist Circumference *P*-value	ΔR^2	Dietary Fish *P*-value	ΔR^2	Dietary Shellfish *P*-value	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

Legend: BMI is the body mass index