Table 2 Percent overlap between top 50 most discriminating masses (based on student's *t*-test) of each discovery project and masses showing $P < 0.05$ in the remaining cohorts

	Genomics Collaborative ($P < 0.05$)	Seracare ($P < 0.05$)	Osaka ($P < 0.05$)
GCI (Top 50)	-	46 (92%)	31 (62%)
Seracare 1 (Top 50)	35 (70%)	-	27 (54%)
Osaka (Top 50)	44 (88%)	47 (94%)	-

eicosatetraenoic acid (**2**) and 8R-Hydroxy-(5Z,9E,11Z,14Z)-eicosatetraenoic acid (**3**), α-tocopherol (**4**) γ-tocopherol (**5**), 13-(6-hydroxy-2,7,8-trimethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid (**6**), 16-(4,5-dimethyl-3,6-dioxo cyclohexa-1,4-dienyl)-2,6,10,14-tetramethylhexadecanoic acid (**7**), 6-hydroxy-2,7-dimethyl-2-(4,8,12-trimethyltridecyl)chroman-8-carbaldehyde (**8**), 6-hydroxy-2,7-dimethyl-2-(4,8,12-trimethyltridecyl)chroman-8-carboxylic acid (**9**), calciferol (**10**), cholecalciferol (**11**), ergosterol (**12**), phylloquinone (**13**), retinol (**14**) and 3β,7α-dihydroxy-5-cholestenoic acid (**15**) (Table 5). The resulting MS/MS data for vitamins A, D, E, K as well as the steroidal molecules (**4** - **15**) showed no similarity to any of the metabolomic biomarkers; for vitamin E type molecules, all had diagnostic fragments characteristic of their chroman rings (m/z 163, 149, 149, 149, 163 and 179 for **4**, **5**, **6**, **7**, **8** and **9** respectively), for vitamin D and analogues, diagnostic fragments formed as a result of the loss of the side chain (m/z 271, 273 and 253, for **10**, **11** and **13**, respectively), for phylloquinone (**13**), the diagnostic fragment m/z 187 for the quinone ring system was prominent, for vitamin A (**14**), the fragment m/z 269 (M + H - H_2O) loses the cyclohexyl ring moiety to form a diagnostic m/z 145 for retinol and for 3β,7α-dihydroxy-5-cholestenoic acid (**15**) the diagnostic retro diels alder fragment at m/z 277 was observed. In addition to this, other carboxylic acid standards with a pregnane ring system, as in **15** (for example, chenodeoxycholic acid and cholic acid), do not show losses of CO_2 upon MS/MS fragmentation (not shown). However, MS/MS fragmentation data of hydroxy fatty acid standards **1**, **2** and **3** (Table 5)

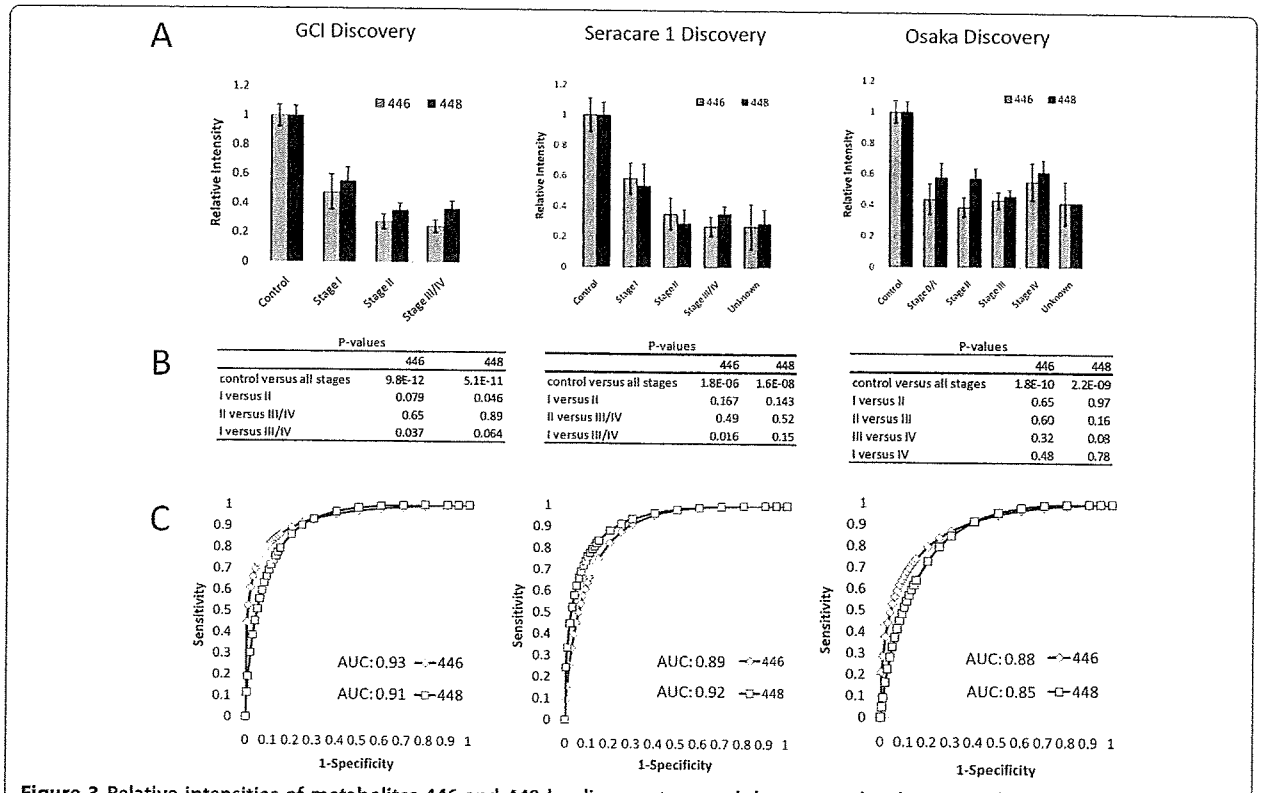

Figure 3 Relative intensities of metabolites 446 and 448 by disease stage and the area under the curves for each discovery dataset. (A) Bar charts of relative intensity versus disease stage in each sample set; (B) summary of *P*-value comparisons between disease stages and controls for metabolites 446 and 448; (C) receiver operating curve analysis based on markers 446 and 448 and all CRCs versus all controls in each discovery set.

Table 3 List of 13 masses detected among the top 50 masses inclusive to all three discovery projects

Rank order	Detected mass	Molecular formula	Part per million	Analysis mode	*P* value	Ratio (CRC/normal)
			GCI			
6	446.3406	C28H46O4	2.22	NAPCI	6.4E-13	0.31
13	448.3563	C28H48O4	2.32	NAPCI	2.5E-12	0.41
8	466.3661	C28H50O5	0.59	NAPCI	9.4E-13	0.25
7	468.3840	C28H52O5	5.39	NAPCI	9.0E-13	0.27
21	492.3829	C30H52O5	2.89	NAPCI	8.5E-11	0.33
24	494.3977	C30H54O5	1.16	NAPCI	1.9E-10	0.35
29	518.3976	C32H54O5	0.92	NAPCI	1.6E-09	0.37
12	538.4259	C32H58O6	4.76	NAPCI	2.5E-12	0.30
44	574.4607	C36H62O5	1.7	NAPCI	1.6E-08	0.40
26	576.4771	C36H64O5	2.99	NAPCI	3.0E-10	0.37
32	578.4931	C36H66O5	3.59	NAPCI	3.2E-09	0.34
11	592.4711	C36H64O6	1.37	NAPCI	2.2E-12	0.27
15	594.4851	C36H66O6	1.41	NAPCI	6.3E-12	0.26
			Seracare			
45	446.3413	C28H46O4	3.79	NAPCI	1.8E-06	0.36
9	448.3570	C28H48O4	3.88	NAPCI	1.6E-08	0.36
3	466.3664	C28H50O5	1.23	NAPCI	8.5E-10	0.34
6	468.3847	C28H52O5	6.89	NAPCI	4.9E-09	0.36
17	492.3835	C30H52O5	4.11	NAPCI	4.6E-08	0.42
34	494.3971	C30H54O5	0.05	NAPCI	6.6E-07	0.41
11	518.3968	C32H54O5	0.63	NAPCI	2.2E-08	0.33
18	538.4263	C32H58O6	5.5	NAPCI	7.8E-08	0.38
32	574.4595	C36H62O5	0.39	NAPCI	6.1E-07	0.32
42	576.4768	C36H64O5	2.47	NAPCI	1.0E-06	0.37
49	578.4933	C36H66O5	3.93	NAPCI	3.2E-06	0.42
30	592.4721	C36H64O6	3.06	NAPCI	5.6E-07	0.27
50	594.4851	C36H66O6	1.41	NAPCI	3.7E-06	0.32
			Osaka			
6	446.3400	C28H46O4	0.87	NESI	1.8E-10	0.44
13	448.3556	C28H48O4	0.76	NESI	2.2E-09	0.54
1	466.3663	C28H50O5	1.02	NESI	2.9E-12	0.50
5	468.3815	C28H52O5	0.05	NESI	1.8E-10	0.49
4	492.3814	C30H52O5	0.15	NESI	7.1E-11	0.57
23	494.3969	C30H54O5	0.45	NESI	2.0E-07	0.62
39	518.3975	C32H54O5	0.72	NAPCI	5.8E-06	0.52
19	538.4237	C32H58O6	0.67	NESI	4.7E-08	0.58
16	574.4600	C36H62O5	0.48	NESI	3.8E-09	0.42
7	576.4756	C36H64O5	0.39	NESI	3.0E-10	0.42
14	578.4910	C36H66O5	0.04	NESI	2.6E-09	0.50
15	592.4703	C36H64O6	0.02	NESI	3.3E-09	0.41
3	594.4859	C36H66O6	0.07	NESI	6.8E-11	0.40

Indicated are the rank order based on *P*-value, detected accurate mass, the computationally predicted molecular formula, the mass difference between the detected mass and mass of the predicted molecular formula in part per million, the mode of analysis (electrospray ionization, ESI; atmospheric pressure chemical ionization, APCI), the *P*-value (based on an unpaired student's *t*-test) between the average peak intensity of control subjects versus colorectal cancer (CRC) patients, and the average peak intensity ratio between CRC patients and controls.

showed peripheral cut ions similar to those produced by MS/MS of the CRC biomarkers, and consistent with what has been described by others for various hydroxylated long-chain fatty acids [29-33]. For example, marker m/z 446 showed peripheral cut ions 427 $[M - H - H_2O]^-$, 401 $[M - H - CO_2]^-$, 409 $[M - H - 2H_2O]^-$, 383 $[M - H - CO_2 - H_2O]^-$ and 365 $[M - H - CO_2 - 2H_2O]^-$ and chain cut ions, 223, 205, 277 as well as others (see Table 5 and Additional File 3). Similar ions were obtained for the other C28, C32 and C36 metabolites

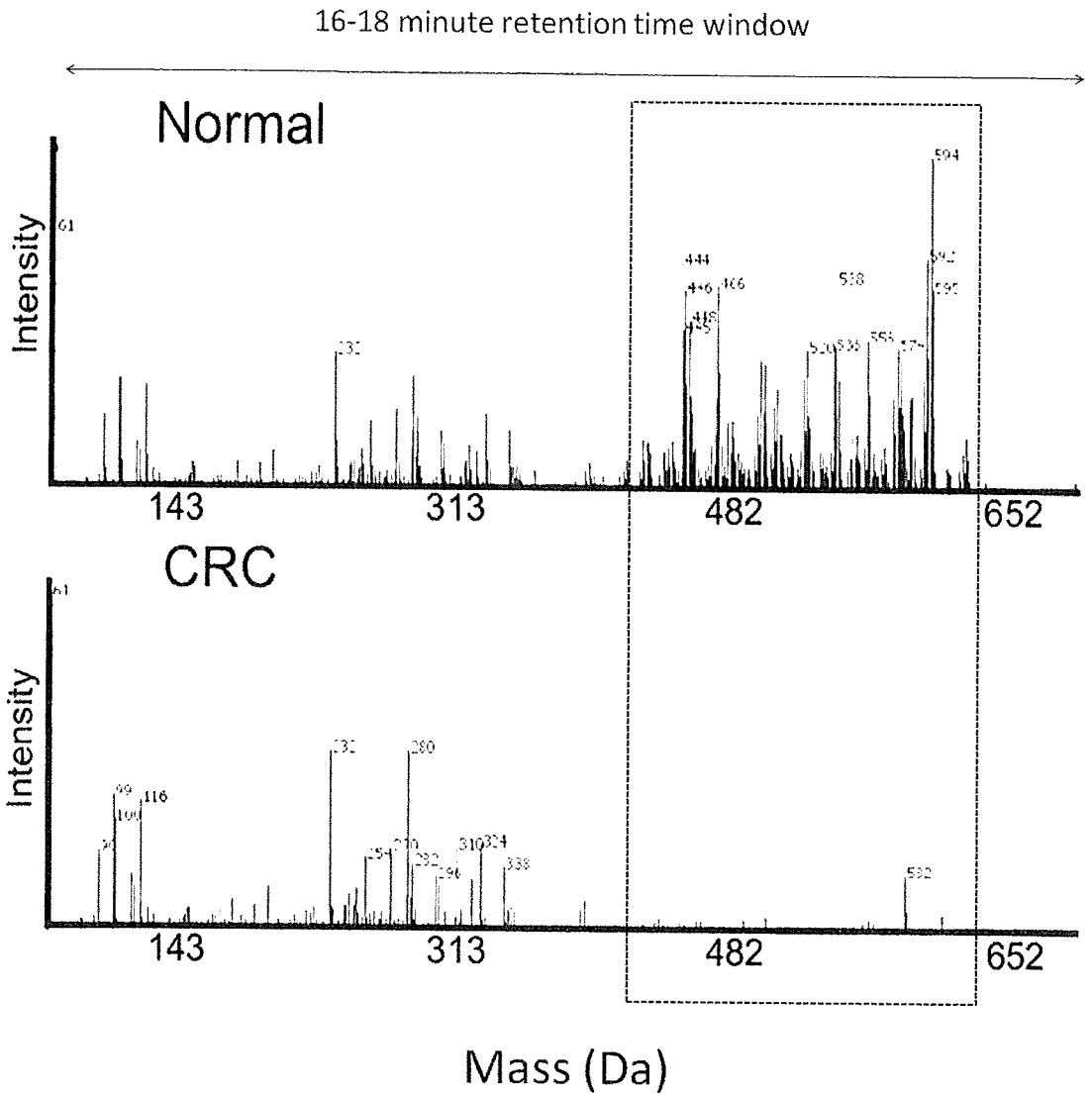

Figure 4 Extracted mass spectrum of serum from normal subjects and colorectal cancer (CRC) patients. Extracts from five representative CRC and five control samples from the Genomics Collaborative discovery set were subject to high performance liquid chromatography followed by full-scan detection on an Applied Biosystems QSTAR XL™ mass spectrometer in atmospheric pressure chemical ionization negative mode. The average intensities of all ions within the mass range 100 to 700 Da eluting between 16 and 18 min are shown for each cohort. The boxed region indicates spectral features present in normal patients but absent from CRC-positive serum.

(Table 4 and Additional File 9). Collectively, these deductions indicated that the metabolomic markers were not analogues of vitamins A, D, E, K and steroids, but rather long-chain hydroxy fatty acids containing varying degrees of unsaturation. We collectively refer to these metabolites as hydroxylated polyunsaturated ultra long-chain fatty acids (hPULCFAs; where the term 'ultra' has been used to refer to C30 and longer chain fatty acids [34]).

Next, an enrichment strategy using bulk serum extracts and a two-stage flash column chromatography approach followed by nuclear magnetic resonance (NMR) analysis was carried out to provide further structural verification of the hPULCFAs. First, reverse phase FCC using a water-acetonitrile solvent gradient was performed and the resulting fractions analysed by LC/MS. Fractions containing the hPULCFAs (fraction 9, Additional Files 10 and 11) were pooled and subjected to normal phase FCC using chloroform-methanol mixtures to obtain an approximately 65% rich semi-purified fraction labelled sample A (Additional File 12). LC and MS/MS analyses (MS2 and MS3) data

on sample A were used to track and confirm enrichment of the markers. NMR (^1H, ^{13}C and 2D) analyses on sample A and its methyl esters revealed resonances and correlations (Table 6) consistent with very long chain polyunsaturated hydroxy fatty acids with observance of some suppression of resonances for hydrogen atoms attached to sp^2 carbons.

Independent validation using MRM methodology

Reduced levels of hPULCFAs in the blood of CRC patients was further confirmed using a MS/MS approach (see methods) in two more independent populations. The approach is based upon the measurement of parent-daughter fragment ion combinations (referred to as MRM) for quantifying analytes [28,35]. We developed an assay to specifically measure semi-quantitatively three of the 28 carbon hPULCFAs with four oxygens (parent masses 446, 448 and 450; C28H46O4, C28H48O4 and C28H50O4, respectively) as described in the methods. The first study comprised 70 treatment-naive CRC subjects and 70 matched controls, all of which were Caucasians from the USA. The [$^{13}C_1$]cholic acid equivalent concentrations of the three 28-carbon hPULCFAs (named according to nominal mass 446, 448 and 450) for each subject are shown in Figure 5A. Significantly lower levels ($P < 0.001$, actual values shown in Figure 5A) of each of the metabolites was observed in treatment-naive CRC-positive subjects compared to controls. ROC analysis resulted in AUCs of 0.87 ± 0.005 for each of the 28-carbon containing hPULCFAs (Figure 5B). Plotting patients by disease stage showed a slight (but not significant) reduction between stage I and III, with stage IV subjects showing the least reduction (Figures 5C and 5D), albeit it only seven subjects. The corresponding average AUCs of the 28-carbon pool by stage were 0.87 for stage I, 0.88 for stage II, 0.94 for stage III and 0.66 for stage IV.

We next used the MRM method to characterize another independent population of CRC and control subjects from Chiba, Japan. Serum from 40 pre-treatment CRC subjects and 40 controls were analysed and a significant reduction was again observed in the CRC-positive group (Figure 6A). The corresponding average AUC for the three metabolites was 0.97 ± 0.014 (Figure 6B). In this study, a significant correlation with stage was observed ($P < 0.05$) for all comparisons between stages I, II and III/IV (Figures 6C and 6D). The AUCs by stage were 0.93 for stage I, 0.97 for stage II and 1.0 for stage III/IV (two stage IVs were grouped with stage III; Figure 6D).

Discussion

We report here on the discovery of novel hydroxylated polyunsaturated ultra long-chain fatty acids containing between 28 and 36 carbons reduced in the serum of CRC patients compared to healthy asymptomatic controls. The utility of non-targeted metabolomics using high resolution FTICR-MS coupled with flow injection technology for biomarker discovery was demonstrated by applying the technology to three independent test populations. In contrast to the 'training/test-set' approach often used by splitting a single sample set in half to validate the performance of biomarkers [36-38], which often relies on complex algorithms (see review [39]) and can result in bias [40], we carried out fully independent discovery analyses on three separate sample sets of matched cases and controls of different ethnic backgrounds collected from multiple sites around the world to ensure a high degree of robustness and minimal chance of sampling bias. Of the top 50 metabolic discriminators discovered in the Osaka set, 44 and 47 of these were also significantly changed in the GCI and Seracare sets, respectively. This remarkable inter-study agreement indicates that not only is non-targeted FTICR-MS technology a reproducible biomarker discovery engine, but that disease-related metabolomic changes can be highly conserved across geographic locations and races. The reduction of hPULCFAs in the serum of CRC patients was further validated by translation of the non-targeted FTICR-MS discovery into a simple targeted TQ-MRM method for three hPULCFAs, which was used on two further independent and ethnically diverse case-control test populations. ROC AUCs generated from the TQ-MRM method on the two validation studies were consistent with those based upon the same fatty acids detected in the three FTICR-MS discovery studies (Figures 3, 5 and 6). In total, five independent study populations collectively comprising 222 treatment-naive CRC patient samples and 220 disease-free asymptomatic controls were evaluated using two different analytical methods. Indeed, the likelihood of the reported association between the reduction of hPULCFAs and CRC being a false positive result across the five independent sets of samples is astronomically low. Meta-analysis was performed on the false positive rates using *Fisher's Inverse Chi-square Method* (*Reject* H_0 *if* $P = -2\sum_{i=1}^{k} \log p_i > C$; p = P-values of five independent samples, k = five different samples, C = upper tail of the chi-square distribution with 2 k degrees of freedom ($X^2_{0.05,10} = 18.31$))[41,42]. Based upon the meta-analysis, the resulting P-values for markers 446 and 448 were more significant than the individual P-values, at 2.96×10^{-47} and 8.11×10^{-49}, respectively. Although there were differences in the median ages between the CRC and control cohorts in two of the studies, there was no statistically significant trend between age and hPULCFA levels within the individual cohorts and we observed no significant difference between hPULCFA concentrations among the controls from the different populations (not shown). We also observed no differences between genders, and although there were slightly higher BMI levels in the control cohorts for the GCI and Seracare 1 cohorts, the BMIs

Table 4 Tandem-mass spectrometry (MS) analysis of selected 28-carbon containing masses

Marker nominal neutral mass		446	448	450	464	466	468
[M-H]-(%)		445 (100%)	447 (52%)	449 (92%)	463 (70%)	465 (100%)	467 (100%)
Chain cut ions (%)		223 (18%)	277 (11%)	171 (7%)	277 (10%)	241 (7%)	187 (12%)
		222 (11%)	239 (5%)	127 (9%)	241 (68%)	223 (3%)	169 (3%)
		207 (3%)	207 (3%)	125 (12%)	223 (15%)	215 (2%)	141 (2%)
		205 (11%)	169 (6%)	113 (38%)	185 (8%)	185 (4%)	113 (4%)
		113 (5%)	113 (25%)		167 (4%)	167 (4%)	
					113 (28%)	113 (7%)	
Peripheral cut ions (%)	Loss of H_2O	427 (50%)	429 (35%)	431 (80%)	445 (46%)	447 (45%)	449 (84%)
	Loss of $2H_2O$	409 (8%)	411 (6%)	413 (13%)	427 (6%)	429 (8%)	431 (10%)
	Loss of CO_2	401 (95%)	403 (100%)	405 (100%)	419 (100%)	421 (45%)	423 (25%)
	Loss of $CO_2$and H_2O	383 (28%)	385 (15%)	387 (32%)	401 (24%)	403 (20%)	405 (13%)
	*Loss of $CO_2$and $2H_2O$	365	367	369	383 (2%)	385 (4%)	387 (3%)
	*Loss of $3H_2O$				409	411	413
Secondary daughter ions (%)		357 (5%)	331 (3%)	307 (5%)	347 (5%)	349 (4%)	349 (1%)
		329 (11%)	305 (3%)	291 (7%)	319 (5%)	321 (2%)	323 (2%)
		261 (3%)	359 (2%)	295 (5%)	295 (6%)	297 (3%)	309 (2%)
		241 (3%)	289 (3%)	281 (5%)	281 (5%)	281 (3%)	297 (6%)
		233 (5%)	245 (3%)	279 (9%)	279 (5%)	279 (15%)	281 (3%)
		207 (11%)	125 (6%)	263 (7%)	267 (5%)	261 (3%)	279 (5%)
		177 (11%)	123 (3%)	261 (5%)	249 (6%)	251 (3%)	269 (5%)
		123 (5%)	121 (3%)	169 (5%)	195 (10%)	195 (2%)	263 (8%)
		109 (11%)	111 (5%)	111 (5%)	141 (1%)	141 (2%)	251 (4%)
		97 (16%)	97 (5%)	97 (8%)	127 (9%)	123 (4%)	243 (2%)
		83 (11%)	59 (3%)	83 (5%)	121 (6%)	113 (5%)	215 (4%)
		59 (11%)		59 (1%)	101 (6%)	101 (3%)	213 (3%)
					97 (4%)	97 (32%)	197 (3%)
					83 (2%)	83 (2%)	125 (4%)
					59 (2%)	59 (2%)	111 (3%)
							98 (2%)
							57 (1%)

*Ions m ay have been obtained from MS^3 experiments

were matched in the second Seracare validation population suggesting the markers are not related to BMI. A prospective analysis of disease-free subjects equally distributed across various age groups is underway specifically to address any potential age or BMI effects in more detail. Overall our results indicate with a high degree of confidence that a reduction in these metabolites is correlated with the presence of CRC.

The FTICR-MS provided resolution sufficient for confident molecular formula predictions based upon accurate mass in conjunction with extraction, ionization, and statistical correlative information. Although multiple elemental compositions were theoretically assignable to given biomarker masses, only formulas having 28 to 32 carbons, and four to six oxygen were consistently assignable to common masses detected in two or three of the discovery sets. Given a high degree of statistical interaction between the sample-to-sample expression profiles of the hPULCFAs (that is, a high degree of correlation between the relative intensities of the markers across subjects) we suspected they were all part of the same metabolic system and should therefore show related compositions. Detection in negative ionization mode also reduced the likelihood that nitrogen was present in any of the compositions. This information in conjunction with tandem mass spectrometry showing prominent losses of water and carbon dioxide enabled the determination of molecular formulas as shown in Table 3 and Additional File 2. A number of candidate classes of molecules theoretically fitting the molecular formula class were easily excluded using tandem MS. For example, we observed no fragments indicative of condensed ring systems such as those in steroids or vitamin D, and no fragments indicative of chroman ring systems such as those observed in the vitamin E tocopherols. Several other classes of molecules including vitamin K and retinol, and bile acids such as cholic acid and 3β,7α-dihydroxy-5-cholestenoic acid also did not

Table 5 Tandem mass spectrometric results of various standards

Standard		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[M-H]-(%)		335 (45%)	319 (100%)	319 (100%)	429 (14%)	415 (28%)	445 (90%)	445 (68%)	429 (100%)	445 (64%)	397 (6%)	385 (2%)	397 (5%)	451 (28%)	287 (1%)	431 (100%)
*Chain cut ions (%)		219 (15%)	219 (80%)	203 (3%)												
		201 (1%)	203 (15%)	163 (25%)												
		115 (100%)	175 (55%)	155 (60%)												
			113 (20%)	127 (10%)												
				111 (5%)												
*Peripheral cut ions (%)	Loss of H_2O	317 (16%)	301 (60%)	301 (55%)			427 (50%)		401 (30%)	401 (28%)	379 (10%)	367 (18%)	379 (5%)		269 (1%)	413 (1%)
	Loss of $2H_2O$	299														
	Loss of CO_2	291 (2%)	275 (20%)	275 (4%)			401 (1%)	401 (35%)								
	Loss of $CO_2$and H_2O	273 (6%)	257 (70%)	257 (80%)												
	*Loss of $CO_2$and $2H_2O$															
	*Loss of $3H_2O$															
Secondary daughter ions (%)		189 (1%)	167 (5%)	291 (1%)	414 (5%)	400 (78%)	295 (70%)	386 (52%)	163 (100%)	386 (43%)	309 (5%)	273 (3%)	295 (5%)	436 (10%)	187 (5%)	399 (100%)
		163 (1%)	149 (2%)	171 (1%)	163 (100%)	175 (13%)	149 (90%)	179 (60%)	135 (20%)	179 (50%)	213 (15%)	259 (25%)	253 (6%)	241 (21%)	173 (10%)	393 (40%)
		145 (2%)	121 (20%)	107 (1%)	135 (8%)	149 (100%)	136 (100%)	135 (100%)	218 (5%)	166 (20%)	201 (30%)	255 (15%)	211 (12%)	227 (62%)	159 (18%)	373 (40%)
		99 (1%)	99 (1%)	59 (1%)		121 (90%)	121 (20%)	107 (25%)	123 (5%)	135 (100%)	173 (37%)	213 (20%)	159 (20%)	223 (48%)	145 (28%)	355 (40%)
		95 (1%)	59 (1%)							122 (25%)	159 (35%)	173 (47%)	161 (15%)	213 (55%)	119 (15%)	337 (20%)
		71 (1%)								107 (25%)	107 (90%)	161 (52%)	147 (20%)	199 (57%)	105 (20%)	223 (40%)
		59 (1%)									81 (68%)	159 (75%)	107 (25%)	187 (100%)	95 (36%)	85 (40%)
											69 (100%)	149 (40%)	105 (15%)	185 (52%)	93 (28%)	
												147 (72%)	95 (38%)	171 (55%)	81 (58%)	

Table 5: Tandem mass spectrometric results of various standards *(Continued)*

	107 (100%)	93 (17%)	71 (86%)	69 (100%)
	81 (82%)	83 (25%)		
		81 (40%)		
		36 (100%)		

*This terminology is specific to fatty acid fragmentation.
5S,6S-(7E,9E,11Z,14Z)-dihydroxyeicosatetraenoic acid (1), 15S-Hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid (2) and 8R-Hydroxy-(5Z,9E,11Z,14Z)-eicosatetraenoic acid (3) (Table 6), α-tocopherol (4) γ-tocopherol (5), 13-(6-hydroxy-2,7,8-trimethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid (6), 16-(4,5-dimethyl-3,6-dioxo cyclohexa-1,4-dienyl)-2,6,10,14-tetramethylhexadecanoic acid (7), 6-hydroxy-2,7-dimethyl-2-(4,8,12-trimethyltridecyl)chroman-8-carbaldehyde (8), 6-hydroxy-2,7-dimethyl-2-(4,8,12-trimethyltridecyl)chroman-8-carboxylic acid (9), calciferol (10), cholecalciferol (11), ergosterol (12), phylloquinone (13), retinol (14) and 3β,7α-dihydroxy-5-cholestenoic acid (15).

Table 6 ^{1}H nuclear magnetic resonance (NMR) data of colorectal cancer (CRC) biomarker pool (sample A) and their methyl esters

Types of protons	CRC biomarker pool	Methyl esters of CRC biomarker pool
CH_3	0.83-0.90	0.83-0.90
CH_2	1.21-1.24, m	1.21-1.24, m
-$CH_2$$CH_2$COOH	1.57-1.65, m	1.53-1.69, m
-CH_2CH=CH-	1.98-2.08, m	1.94-2.03, m
CH_2COO	2.23-2.28, m	2.23-2.31, m
-CH=CH-CH_2-CH=	2.75-2.79, m	2.74-2.82, m
OCH_3	-	3.64, s
-CH(OH)CH=	3.45-3.71, 4.03-4.26	4.02-4.12, 4.16-4.26, 4.58-4.60
-CH=	5.10-5.47, m	5.08-5.40, m
-CH(OH)CH=	5.76-5.91, m	5.75-5.90, m

*NMR solvent is $CDCl_3$, signals assigned using 2D NMR experiments (HMQC and HMBC)

show comparable fragmentation patterns. However, the similarity in fragmentation pattern, particularly in the relative abundances of daughter ions resulting from losses of CO_2 and H_2O, and chain cut ions from the hPULCFAs to known hydroxy fatty acid standards as well as other fatty acids reported in the literature such as the resolvins and protectins (discussed below), allowed for the identification of the metabolites as hydroxylated polyunsaturated ultra long-chain fatty acids. Examination of the MS/MS data for the C28 series (masses 446, 448, 450, 464, 466 and 448) revealed a consistent 113 Da daughter ion, which we conjecture to represent the carboxy-terminus chain fragment -CH_2-CH=CH-CH_2-CH_2-COOH. In addition, a consistent loss of 54 (-CH=CH-CH_2-CH_2-) from the [M-(CO_2+H_2O)] daughter ion was observed for the 446, 448, 464 and 466, but not the 450 and 468 molecules, suggesting that (1) the 450 and 468 may have a saturated carboxy terminal region and (2), that there are likely no hydroxyl moieties within this region of the molecule. MS/MS data of all the C28 and other markers also did not show the diagnostic fragment obtained with a 1,2-diol motif as observed for **1** (base peak is chain cut ion at m/z 115) and NMR on fractions enriched via flash-column chromatography showed lower than expected integration values obtained for the ^{1}H NMR signals at δ 2.78 (methylene interruptions between double bond carbons) and at δ 5.12 - 5.90 (hydrogen atoms on double bond carbons). Cumulatively these results suggested that the hydroxyl groups in the molecules are likely bonded to the carbon atoms between the sp^2 carbons at least seven carbons from the carboxy end. Confirmation of the exact positions of the hydroxyl groups and precise locations of unsaturations in individual hPULCFAs using

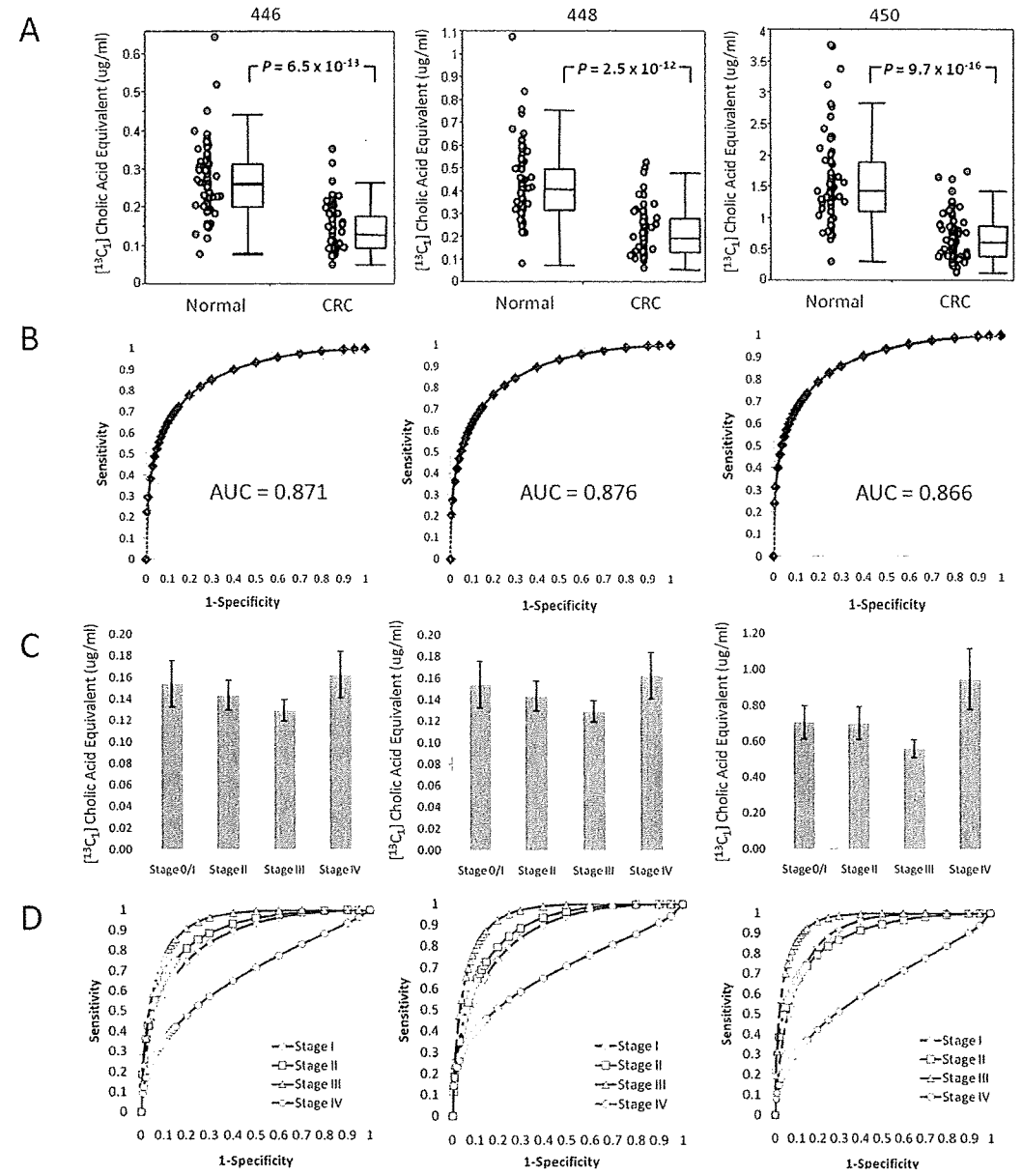

Figure 5 Results of triple-quadrupole multiple reaction monitoring analysis of the Seracare 2 validation sample set. (A) Scatter plots of the concentrations of hydroxylated polyunsaturated ultra long chain fatty acids (hPULCFAs) 446, 448 and 450 expressed as [$^{13}C_1$]-cholic acid equivalents in asymptomatic normal controls and pre-treatment colorectal cancer patients, (B) receiver operating curve (ROC) analysis based upon the corresponding scatter plots in (A). Grey dotted lines indicate the 95% confidence interval. (C) Bar charts of the average concentration equivalents of hPULCFAs by disease stage. Error bars represent standard errors of the mean. (D) ROC analysis by disease stage.

preparatory HPLC and chemical synthesis is in progress and will be reported in subsequent publications.

Interestingly, the metabolite markers reported in this study represent a human-specific metabolic system. We analysed serum samples from multiple species, including rat, mouse and bovine, as well as multiple different sample sources including numerous cell lines, conditioned media, tumour and normal colonic tissue from patients in the GCI discovery set, and brain, liver, adipose and other tissues from various species, all of which failed to show any detectable levels of these hPULCFAs (results not shown). We also could not detect these molecules in various plant tissues or grains, including policosanol extracts which are rich in saturated C28 and longer-chain fatty acids [43,44]. This suggests that the molecules may originate from human-specific metabolic

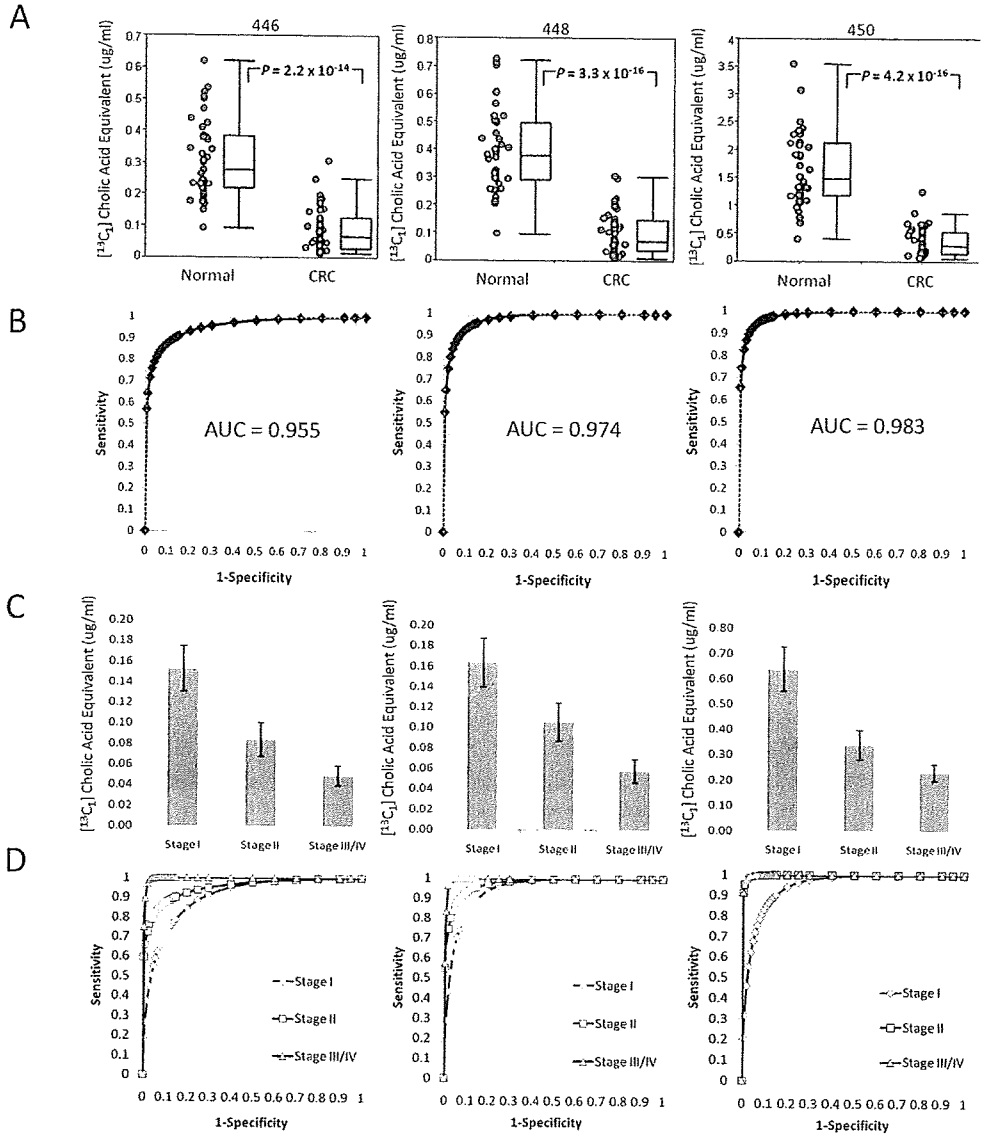

Figure 6 Results of triple-quadrupole multiple reaction monitoring analysis of the Chiba validation sample set. (A) Scatter plots of the concentrations of hydroxylated polyunsaturated ultra long-chain fatty acids (hPULCFAs) 446, 448 and 450 expressed as [$^{13}C_1$]-cholic acid equivalents in asymptomatic normal controls, and pre-treatment colorectal cancer patients, (B) receiver operating curve (ROC) analysis based upon the corresponding scatter plots in (A). Grey dotted lines indicate the 95% confidence interval. (C) Bar charts of the average concentration equivalents of hPULCFAs by disease stage. Error bars represent standard errors of the mean. (D) ROC analysis by disease stage.

processes, such as specific p450-mediated and/or microbiotic processes. The lack of detection in tumour or normal colonic tissue suggests that the metabolites are not 'tumour-derived markers' and, combined with the high rate of association in stage I cancer, it is not likely that the reduction is the result of tumour burden. Analysis of post-surgery samples is currently in progress to address this question. However, the further reduction of levels observed in some late stage Japanese cases (Figure 6) could be explained if lower levels of the hPULCFAs were indeed indicative of progression rate in this group. It is also important to note that in all control groups reported in this paper, subjects were not colonoscopy-confirmed to be free of tumours or advanced neoplasia. Based upon colonoscopy results by Collins *et al* in average-risk subjects, up to 10% of an asymptomatic population can be positive for advanced neoplasia [45]. Therefore, the ability of these metabolites to discriminate between subjects at risk and not at risk for CRC is likely under-estimated in our results. Studies are

currently in progress to evaluate endoscopy-confirmed controls, to assess the effect of treatment on the markers, and to investigate any possible association with various grades of colon pathologies and non-malignant GI disorders as well as other cancers.

Although fatty acids of this length containing hydroxyl groups have never been reported as far as we are aware, they appear to resemble a class of hydroxylated very long-chain fatty acids knows as the resolvins and protectins that originate from the n3 essential fatty acids EPA and DHA, respectively, which are critical in promoting the resolution of acute inflammation. The inability to sufficiently 'resolve' acute inflammation is the leading theory behind the establishment of chronic inflammatory states which underlie multiple conditions including cancer [46] and Alzheimer's Disease [47]. Of particular relevance is the effect of pro-resolution long-chain hydroxy fatty acid mediators on intestinal inflammatory conditions such as irritable bowl disease (IBD), Crohn's Disease, Colitis and colon cancer. Both Resolvin E1 (RvE1) and Lipoxin A4 (LXA4) have been implicated with protective effects against colonic inflammation. RvE1 was shown to protect against the development of 2,4,6-trinitrobenze sulphonic acid-induced colitis in mice, accompanied by a block in leukocyte infiltration, decreased proinflammatory gene expression, induced nitric oxide synthase, with improvements in survival rates and sustained body weight [48]. Similarly, LXA4 analogues have been shown to attenuate chemokine secretion in human colon *ex vivo* [49], and attenuated 50% of genes, particularly those regulated by NFκB induced in response to pathogenically induced gastroenteritis [50]. *In vivo*, LXA4 analogues reduced intestinal inflammation in DSS-induced inflammatory colitis, resulting in significantly reduced weight loss, haematochezia and mortality [50]. Structurally, resolvins and protectins (as well the n6 lipoxins) comprise mono-, di- and tri-hydroxylated products of the parent VLCFAs, catalyzed by various lipoxygenases, cyclooxygenases and p450 enzymes [51-55]. The possibility that the hPULCFAs reported here represent elongation products of these molecules cannot be excluded. Future studies will be required to address the origin, as well as the biological role, if any, that these molecules may play in defending the body against CRC development.

Although we report results from multiple case-control cohorts each having a limited sample size, the average AUC across all the samples reported here was 0.91 ± 0.04, which translates into approximately 75% sensitivity at 90% specificity with little to no disease-stage bias. The real-world screening performance is currently being evaluated through two large ethically approved prospective clinical trials, one in collaboration with the Saskatchewan Cancer Agency and the Saskatchewan Provincial Government (PDI-CT-1; n = 5000), and the other with the University of Calgary (PDI-CT-3 n = 1500). Clinically relevant questions are being addressed, including correlation between hPULCFAs and CRC in a prospective hospital screening environment, correlation with other non-malignant gastrointestinal disorders (such as IBD, Crohn's and colitis), whether there is any correlation with various stages of neoplasia or polyps and family history and whether subjects with low hPULCFA levels show higher incidence rates of CRC than subjects with 'normal' levels over time.

In summary, we have identified a consistent reduction of novel circulating hPULCFAs in CRC patients which could have considerable implications for CRC diagnosis and screening and possibly prevention and treatment. Adherence to currently recommended screening modalities, namely faecal occult blood testing and colonoscopy, is poor due to a number of factors including public acceptance, risk, cost and available resources. The use of a serum-based test to screen the population for subjects who are high risk would focus endoscopy resources on subjects who need it the most, resulting in a higher detection rate, particularly in early stages of the disease. Given the positive prognosis of early-stage therapeutic intervention, it is tempting to speculate that hPULCFA-based screening could one day result in decreased CRC mortality.

Conclusions

We have shown that comprehensive non-targeted metabolomics technology based upon high-resolution FTICR mass spectrometry represents a powerful and robust approach for small-molecule biomarker-driven discovery. Accurate mass measurements combined with conventional MS/MS resulted in the rapid identification of key structural characteristics of the novel metabolites discovered and the assignment of putative chemical structures. The subsequent translation of these metabolite biomarker discoveries into an efficient and clinically viable high-throughput semi-quantitative triple-quadrupole platform represents a significant advancement in the clinical implementation of biomarker discoveries. The reduction of systemic hydroxylated ultra-long chain fatty acids in CRC patients raises intriguing biological and aetiological questions given the large numbers of sporadic CRC cases and the heavy influence of lifestyle and diet on risk. Further research is ongoing regarding the potential role(s) these novel molecules play in CRC progression and whether they have any association with previously established risk factors.

Additional file 1: Fourier transform ion cyclotron resonance mass spectrometry feature data.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S1.XLSX]

Additional file 2: Top 50 discriminating masses (based on student's *t*-test) of each discovery project. Masses shaded grey were detected in the top 50 in two of the three studies. Indicated are the detected accurate mass, the computationally predicted molecular formula (for masses shaded in grey), the mass difference between the detected mass and mass of the predicted molecular formula in part per million (ppm), the mode of analysis (electrospray ionization; atmospheric pressure chemical ionization), the *P*-value (based on an unpaired student's *t*-test) between the average peak intensity of control subjects versus colorectal cancer (CRC) patients and the average peak intensity ratio between CRC patients and controls.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S2.PNG]

Additional file 3: Tandem mass spectrometry spectra for biomarker m/z 446.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S3.PNG]

Additional file 4: Tandem mass spectrometry spectra for biomarker m/z 448.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S4.PNG]

Additional file 5: Tandem mass spectrometry spectra for biomarker m/z 450.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S5.PNG]

Additional file 6: Tandem mass spectrometry spectra for biomarker m/z 464.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S6.PNG]

Additional file 7: Tandem mass spectrometry spectra for biomarker m/z 466.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S7.PNG]

Additional file 8: Tandem mass spectrometry spectra for biomarker m/z 468.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S8.PNG]

Additional file 9: Tandem mass spectrometry of hydroxylated polyunsaturated ultra long-chain fatty acids.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S9.PNG]

Additional file 10: Purification process to obtain hydroxylated polyunsaturated ultra long-chain fatty acids (hPULCFA) enriched fractions from human serum. Dried organic extracts of serum were initially purified in a reversed phase flash column chromatography using water/acetonitrile step solvent gradient to obtain semi purified hPULCFA enriched fraction (F9). Several of F9s were combined for a secondary purification step in a normal phase flash column chromatography using hexane/chloroform/methanol step solvent gradient to obtain highly hPULCFA enriched fraction 7 (F7_2).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S10.PNG]

Additional file 11: Liquid chromatography/mass spectrometry spectra of Stage I fraction 9 (F9) containing a mixture of fatty acids and colorectal cancer biomarkers obtained after fractionating serum extract on reverse phase column.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S11.PNG]

Additional file 12: Liquid chromatography/mass spectrometry spectra of Stage II fraction 7 (F7) containing approximately 65% enrichment of hPULCFAs.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-8-13-S12.PNG]

Abbreviations

APC: adenomatous polyposis coli; APCI: atmospheric pressure chemical ionization; AUC: area under the curve; BuOH: butanol; CRC: colorectal cancer; CUR: curtain gas; ESI: electrospray ionization; EtOAc: ethylacetate; FCC: flash column chromatography; FTICR-MS: Fourier transform ion cyclotron resonance mass spectrometry; GS: gas source; HPLC: high performance liquid chromatography; hPULCFA: hydroxylated polyunsaturated ultra long-chain fatty acid; LC: liquid chromatography; LXA4: lipoxin A4; MRM: multiple reaction monitoring; MS/MS: tandem mass spectrometry; mz: mass to charge ratio; NC: nebulizer current; NMR: nuclear magnetic resonance; ppm: part per million; Q-TOF: quadrupole time-of-flight; ROC: receiver-operator characteristic; RvE1: resolving E1; SELDI: surface-enhanced laser desorption ionization; TLC: thin layer chromatography; TQ-MRM: triple-quadrupole MRM; VLCFA: very long-chain fatty acid.

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Authors' contributions

SAR was the group leader, lead author and writer, performed the study design and sample selection, completed all the discovery FTICR-MS data analysis, including statistical analysis, supervised the structural elucidation, MSMS interpretation and overall experimental designs. PWAK performed MS/MS and NMR analysis and interpretation and analysed all comparison MSMS standard data. DJ performed MS/MS and fraction column chromatography enrichment and NMR interpretation. DH developed FTICR-MS analytical methods, including extraction protocols, and helped to develop the TQ-MRM methodology used. JL developed high performance liquid chromatographic methods and analysed samples for HPLC-TOF analysis. YL developed and optimized TQ-MRM methods and analysed validation sample sets. WJ performed and optimized HPLC-TOF and MS/MS analysis and samples, including enriched serum extracts. AK was involved in the optimization of the TQ-MRM methodology, QAQC and analysis of validation (Seracare and Chiba) samples. YY developed the original chromatographic (HPLC) methods used to isolate the hPULCFAs and aided in the experimental design of the Chiba and Osaka sample sets. AMK oversaw the structural elucidation processes including NMR interpretation and MS/MS interpretation. MH aided in MS/MS and NMR data generation and interpretation. KKSM was involved in the biostatistical analysis, including the analysis of HTS data, Fisher analysis and all SAS work. PLW was involved in the interpretation of MS/MS data, NMR data, mechanistic insights into MSMS fragmentation patterns and proposed structural motifs. KK was involved in the sample selection and study design for the genomics collaborative and Seracare Lifesciences sample sets, the selection of matched cases and controls, clinical data organization and verification of disease pathology. IT was involved in the design of clinical trials in Osaka, patient selection and pathology confirmation. MM was involved in the clinical trial design in Osaka, patient selection and discovery analysis, clinical staging, pre-versus post surgery data collection and analysis. MS was the Head of the lower gastroenterological surgery group, Osaka, responsible for patient recruitment and trial design. MM was the group leader at the Osaka Department of

Surgery and was involved in the experimental design of the Osaka discovery sample set, data analysis/interpretation and unblinding of data. HM was the head of surgery (for CRC population, Chiba) responsible for the patient enrolment and selection for the Chiba samples. FN was the group leader at Chiba overseeing the entire project, including protocol design and approvals, data analysis and unblinding. DBG was the President and CEO of Phenomenome Discoveries Inc, and oversaw most of the efforts at PDI, was integrally involved in the interpretation of MS/MS data, the development of FTICR methodology, the experimental designs and was also a significant contributor to the format and direction of the manuscript.

Competing interests

The following authors are full-time employees and have received salaries from Phenomenome Discoveries Inc, Saskatoon, Canada: Shawn A Ritchie, Pearson W K Ahiahonu, Dushmanthi Jayasinghe, Doug Heath, Jun Liu, Yingshen Lu, Wei Jin, Amir Kavianpour, Yasuyo Yamazaki, Amin M. Khan, Mohammad Hossain, Khine Khine Su-Myat and Paul L Wood. Dayan B Goodenowe is the co-founder, President and CEO, of Phenomenome Discoveries, Inc. Only Dayan B Goodenowe owns shares in the company. Kevin Krenitsky, Ichiro Takemasa, Masakazu Miyake, Mitsugo Sekimoto, Morito Monden, Hisahiro Matsubara and Fumio Numura have no competing financial interests. None of the authors have non-financial competing interests. Phenomenome Discoveries Inc is financing the article processing fees for the manuscript. Shawn A Ritchie and Dayan B Goodenowe are named inventors on submitted patent applications relating to the discoveries disclosed within the manuscript and have both received a salary from Phenomenome Discoveries Inc (the organization named in the patents).

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