Supplementary Material

Serum Lipidomic study reveals potential early biomarkers for predicting response to chemoradiotherapy in advanced rectal cancer: a pilot study

Material and Methods

Patients

Between March 2013 and September 2014, 18 patients with primary LARC were treated with preoperative CRT. All patients underwent initial workups for clinical staging, including physical examination, chemistry tests, colonoscopy, abdomino-pelvic computed tomography (CT), and morphological plus functional rectal magnetic resonance imaging (MRI + DWI)[1]. All patients presented a locally advanced disease (clinically TNM stage II and III) evaluated by imaging, as previously described, and a histopathologically proven rectal adenocarcinoma (as reported in Table S1). For four patients, treatment consisted in preoperative radiotherapy to the pelvis at a dose of 45 Gy in 25 fractions, followed by a 5.4-Gy boost to the primary tumour within 6 weeks, associated with chemotherapy (Capecitabine and Oxaliplatin). The other 14 patients received radiotherapy to the pelvis (45 Gy in 25 fractions) with a concomitant 10 Gy boost (twice weekly) to the primary tumour within 5 weeks, associated with Capecitabine alone. Patients underwent curative TME at 8^{th}–10^{th} week after completion of CRT; before surgery all patients were re-staged according to the initial imaging procedures (Colonscopy, CT and MRI + DWI). The tumor response was assessed in TME specimens by pathological examination, according to Mandard's tumor regression grading (TRG) system: TRG 1 and TRG 2 scores were considered RP, whereas TRG 3, TRG4, and TRG5 scores were classified as NRP[2]. This protocol was approved by the institutional review board; all patients were informed about the procedures and provided written informed consent to participate in the study. In order to protect human subject identity a number code was employed for specimen identification.

Sample collection

Sera were prospectively collected during routine chemistry tests before treatment (t0) and at day 14° (t14) and 28° (t28) of CRT, using Vacutainer with plain red top cup

(Anamedica) for venipuncture (glass tube). All samples were maintained at room temperature (23±1 °C) for 45 min to allow sample coagulation and centrifuged at 4 °C for 15 min at 1400 g. Serum was aspirated, avoiding the fluid immediately above the buffy coat layer, and 2 ml of serum was collected in polypropylene tubes, divided into aliquots and snap frozen at −80 °C.

Lipid extraction procedure

Lipid extraction was performed on 100 µL of serum from each patients. Serum samples were fortified by adding 10 µL of a solution of Lysosphingomielin (LSM) at known concentration of 100 µg/mL, used as Internal Standard (IS). After vortexing, 300 µL of methanol was added for protein precipitation. The obtained solution was vortexed, mixed for 5 minutes at 10°C and centrifuged for 15 minutes at 20880 rcf (T=10°C). After centrifugation 1 mL of MTBE (methylterbutylether) and 250 µL of water were added to supernatant (350µL), vortexed, mixed and centrifuged at the same conditions previously described. The upper MTBE phase was dried and finally dissolved in 100 µL of ACN (Acetonitrile), vortexed and centrifuged for 15 minutes at 20880 rcf (T=20°C). Ninety microliters of this solution were recovered and 40 µL was transferred in the vials for LC-MS/MS analysis. The remaining 50 µL was stored at -80°C for further analyses.

LC-MS/MS lipid profiling

The LC-MS/MS method was performed partially following the method described by Xiong et al. [3] Briefly, after extraction procedure, the total lipid extract was injected (20 µL) and separated by HPLC (Waters). In our study, samples from each group were alternated in random order in a single analysis batch. Elution at 200 µL/min was obtained using a column Atlantis HILIC (Waters) silica 3µm, 150mm x 2.1mm. Separation was performed using a gradient of formic acid 0.1% (solvent A) and acetonitrile (solvent B) as follow: 92% B for 5 minutes; to 70% B in 15 minutes; isocratic 70%B for 2 minutes; to 35% B in 22 minutes; finally re-equilibration in 27 minutes. The method was developed and optimized using eight standards polar lipid (Avanti Polar Lipid,Inc.): Phosphatidylcholine(14:0/14:0), Lysophosphatidylcholine(14:0/0:0), Sphingomyelin (d18:1/16:0),Sphingomyelin(d18:1/0:0), Phophatidylethanolamine(14:0/14:0), Lysophosphatidylethanolamine(16:0/0:0), Phosphatidylserine(18:0/18:0), Phosphatidylglycerol(16:0/16:0) (10 µg/mL). The LC system was coupled on-line with

a triple quadrupole (Quattro Ultima Platinum Micromass, Waters) through an ESI source operating in positive ion mode. A 3.5 kV tension was applied on the capillary while a 60 V tension was applied on the cone. The profile of biological phospholipids was performed by MS/MS fragmentation functions performed by argon as collision gas. For the detection of several subclasses in a single analysis, data acquisition was performed through four different MS/MS functions, as shown in Table S2, and as already reported in the paper by Xiong et al.

Method Assessment

A working solution containing 10ug/ml of each standards (PC(14:0/14:0), LPC(14:0), SM(D18:1/16:0), LSM(d18:1), PE(14:0/14:0), LPE(16:0), PG(16:0/16:0), and PS(18:0/18:0)) was analyzed during the entire analytical section to test the reproducibility of the method. LC-MS/MS extracted ion chromatograms, obtained by a single analytical run of standards, are reported in Figure S1. Table S3 shows the reproducibility of the retention time and peaks area for every compound. Results indicated a CV% related to the retention time lower than 3.5% (n=10), CV% related to peaks area lower than 19.13% (n=10). Statistical data were obtained by excluding outliers, estimated by the software GraphPad QuikCalcs (http://graphpad.com/quickcalcs/Grubbs1.cfm).

Lipidomics data processing

The acquired LC-MS/MS data were processed, for each MS/MS function, using MarkerLynx (Waters, UK). This procedure allowed deconvolution alignment, and data reduction to give a table of mass and relative retention time pairs with associated relative intensities for all the detected peaks.The mass tolerance was set at 0.5 Da; the peak width at 5% height and the peak-to-peak baseline noise were calculated automatically by the software; the mass window was set at 0.5 Da; retention time window was set at 0.4 minute; the noise elimination level was 6; the minimum intensity (as a percentage of the BPI) was set at 1%.The obtained Markerlynx data were exported in a compatible format for the multivariate analysis with Simca-P+ 11.0 software (Umetrics AB, Umeå, Sweden).

LC-MS/MS targeted analysis

The interesting potential biomarkers highlighted have undergone to LC-MS/MS targeted approach. These lipids were analyzed in Multiple Reaction Monitoring (MRM) acquisition mode, by using the same chromatographic and the mass spectrometry conditions described for the lipidomic profiling. Functions 1-4 (Table S4) refer to the MRM experiment created for each lipid of interest. Data processing and quantification were performed using the QuanLynx software (Waters) provided with the instrument. Data obtained were rescaled in respect to IS added to the each sample (reported in bold character in Table S4).

Statistics

The four data matrix obtained by MarkerLynx were used for partial least squares discriminant analysis (PLS-DA) using SIMCA-P+. In order to find differential circulating lipids in sera analyzed, a parameter named VIP (Variable Importance in the Projection) was employed to reflect the variable importance in the discriminant analysis. The major discriminant variables were selected and underwent to D'Agostino and Pearson omnibus normality test, in order to determine the normality of each variable measured in each group. When normality was accepted, the Student's t-test was employed, otherwise the Mann Whitney U-test was used for comparing the groups, by using GraphPad Prism (GraphPad software, Inc. USA). Finally, the significantly different variables were tentatively identified by Lipidmaps Database and Human Metabolome Database (HMDB), and by using the MS/MS data and the retention time. Heatmap and ROC curve analysis were performed by Metaboanalyst 3.0 without any pre-processing manipulation (http://www.metaboanalyst.ca/faces/home.xhtml) [4].

Supplemental figures:

Figure S1:LC-MS/MS extracted ion chromatograms of a mixture of standards obtained through four different MS/MS functions in a single analytical run. Panel A: parent scan of 184 m/z; panel B: neutral loss of 141 m/z; panel C: neutral loss of 172 m/z; panel D: neutral loss of 185 m/z. It has been reported a representative mass spectrum for each chromatogram (Panel 1,2,3,4). PSM (Palmitoyl Sphingomyelin), DMPE (dimyristoyl phosphatidylethanolamine), DPPG (dipalmitoyl phosphatidylglycerol), DSPS (distearoyl phosphatidyl serine)

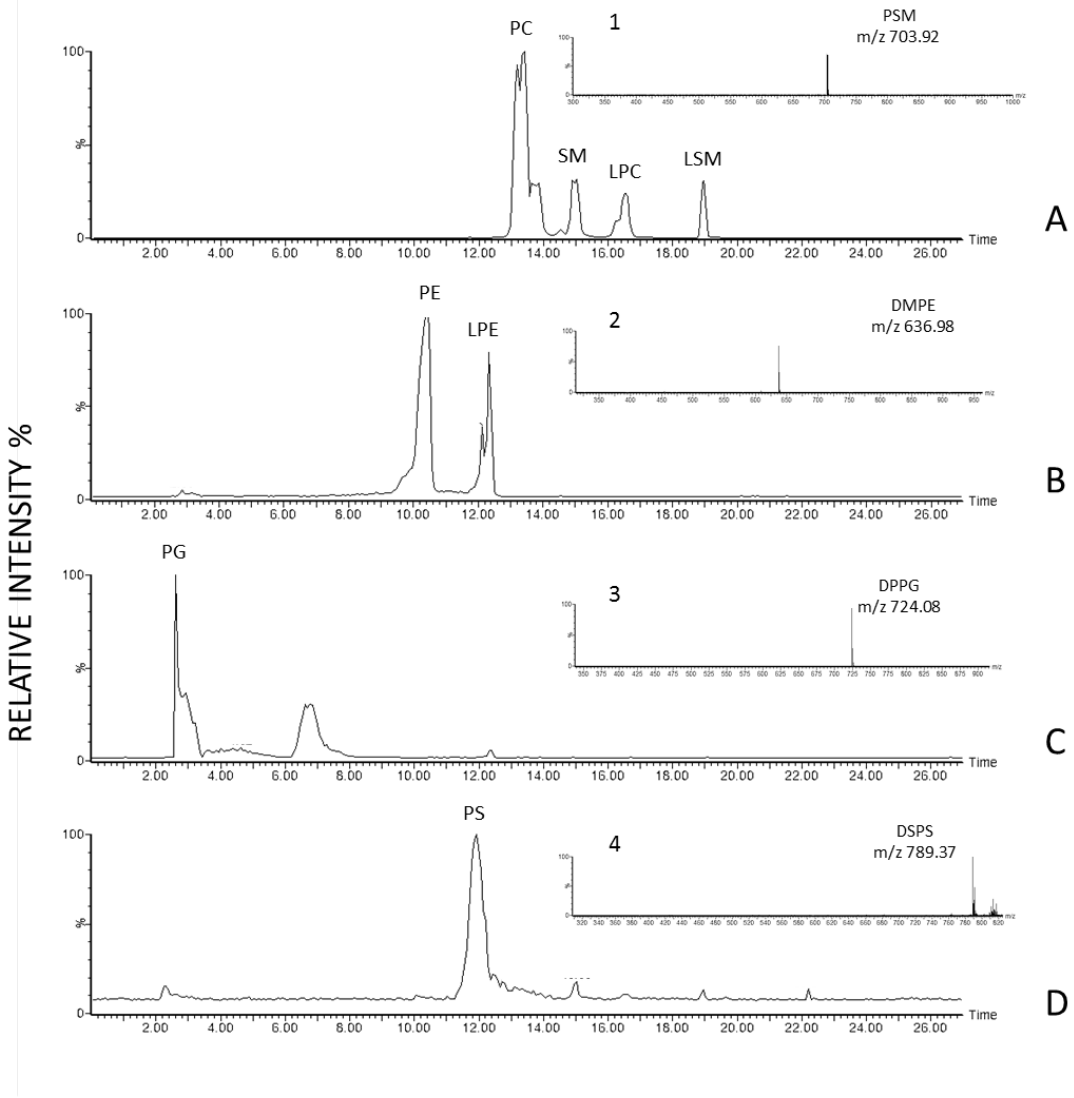

RELATIVE INTENSITY %
PC
1
PSM
m/z 703.92
SM
LPC
LSM
A
PE
LPE
2
DMPE
m/z 636.98
B
PG
3
DPPG
m/z 724.08
C
PS
4
DSPS
m/z 789.37
D
Time

Figure S2: PCA score plots based on the data from LC-MS/MS lipids profiling containing all acquired data for PCs/SMs class. Blank Sample (blue); mixture of standards (green); RP (black); NRP (red).

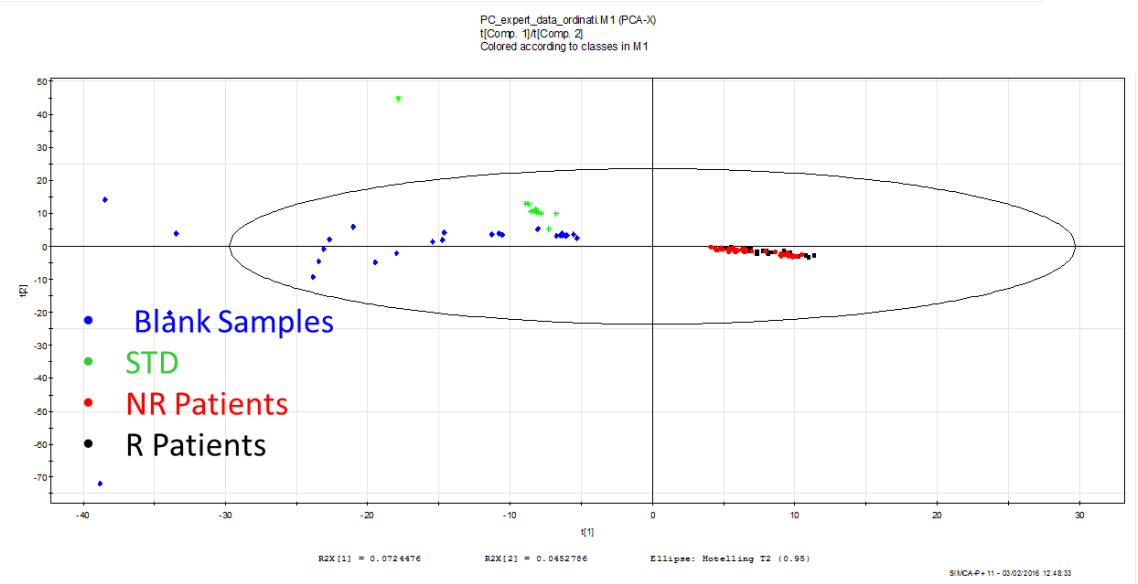
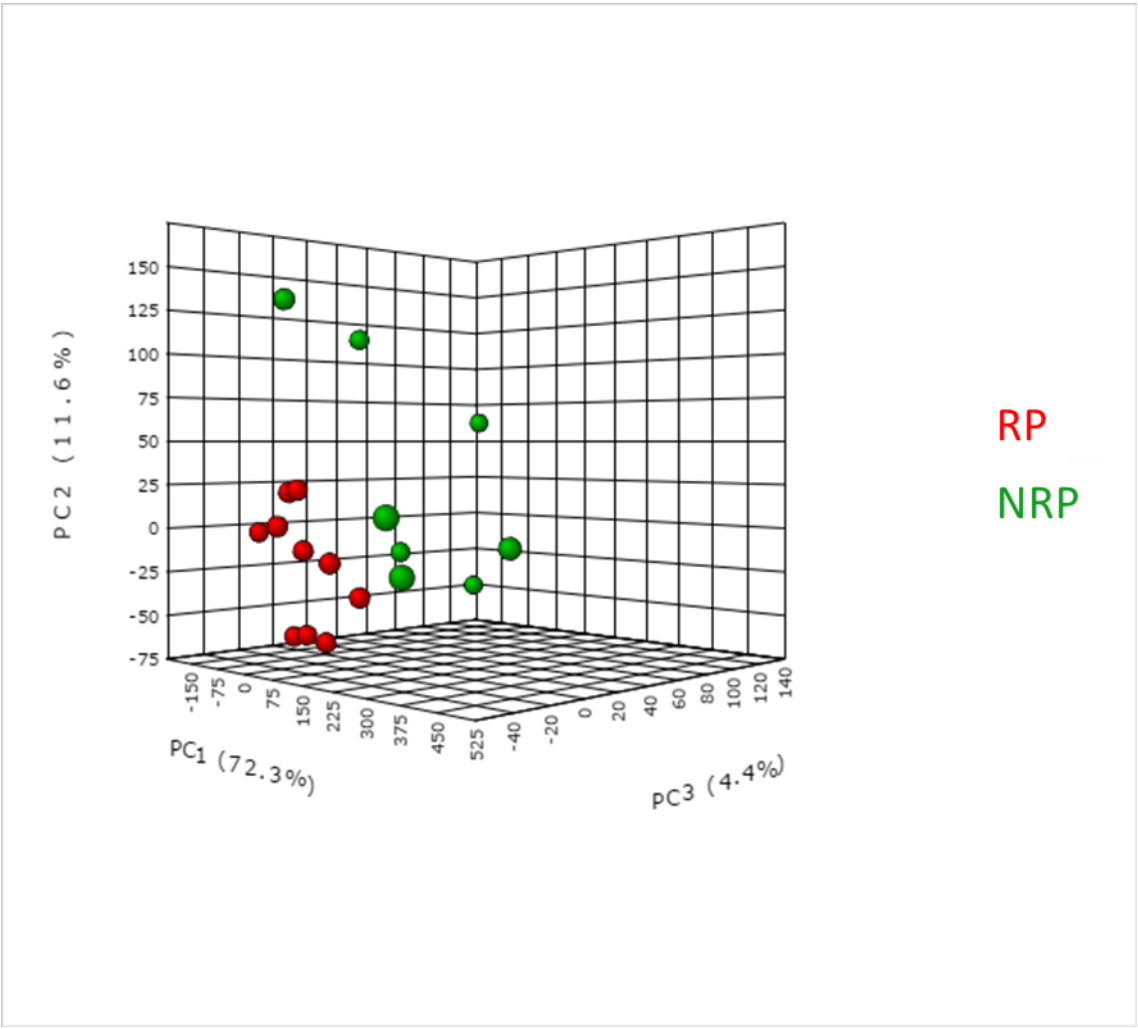


Figure S3

Figure shows the 3D score plot for the PCA analysis by using LC-MS/MS data from RP and NR patients. The RED circle are the RP patients, while the green dots are the NRP.



Supplemental tables:

Table S1: Clinical parameters of each patients (RP and NRP). OXA§:Oxalilplatino 130 MG/MQ/ GG1-19-38; LDH: Lactic dehydrogenase; CAPE*: Capecitabine 1300 MG/MQ/DIE; CAPE**: Capecitabine 1650 MG/MQ/DIE; ASA: Acetylsalycil Acid; TRG: Tumor regression grade; ENP: Enalapril; LPZ: Lansoprazole; RNT: Ranitidine; CBZ: Carbamazepine; QTP: Quetiapine; PE: Perindopril; IDP: Indapamide; HCTZ: Hydrochlorothiazide; Dox: Doxazosin; #: Classification of Malignant Tumors (TNM).

Code	TRG	AGE	Creatinine (mg/dL)	Tot. Pt (g/dl)	Glycemia (mg/dl)	Neutrophils (Ux10^3/uL)	LymPhocytes (Ux10^3/uL)	Monocytes (Ux10^3/uL)	Eosinophils (Ux10^3/uL)	Basophils (Ux10^3/uL)	GOT (U/L)	GPT (U/L)	LDH (U/L)	Clinical T #	Clinical N #	Clinical Stage #	Grade	Basal Therapy	Chemotherapy	TOX GU (sec RTOG)	TOX GI (sec RTOG)
NRP_4	4	55	0,8	6,6	83	7,23	2,94	0,71	0,25	0,07	19	35	376	3	1	IIIB	G3	-	CAPE **	G0	G1
NRP_6	3	66	0,78	6,3	135	2,96	1,29	0,24	0,45	0,01	20	14	443	3	1	IIIB	G2	Acarbose; omegasol	CAPE *+ OXA§	G0	G3
NRP_7	4	57	0,7	7,5	88	4,36	1,8	0,37	0,14	0,04	22	49	527	3	1	IIIB	G2	ASA; esomepraole	CAPE**	G0	G0
NRP_11	4	53	0,9	7,2	98	4,23	1,6	0,5	0,13	0,01	15	26	454	3	2	IIIB	G2	Nebivolon	CAPE **	G0	G0
NRP_13	3	73	1,99	7,2	86	4,82	1,48	0,73	0,82	0,09	32	41	487	3	2	IIIB	G2	Clopidogrel+ ASA; RNT; Bisoprolol	CAPE**	G0	G1
NRP_14	3	72	1,04	6,5	83	4,25	1,35	0,4	0,35	0,02	31	20	416	3	2	IIIB	G2	LPZ; ASA; PE; Alfuzosin	CAPE**	G0	G0
NRP_15	3	47	0,6	7,6	81	2,39	0,97	0,25	0,09	0,02	18	36	543	3	1	IIIB	G2	-	CAPE**	G2	G2
NRP_16	3	67	0,66	7,2	90	3,73	1,81	0,28	0,06	0,01	25	31	626	3	1	IIIB	G2	CBZ; QTP	CAPE **	G0	G2
NRP_18	3	69	-	7,7	93	3,73	1,31	0,56	0,09	0,01	36	46	742	3	1	IIIB		-	CAPE**	G0	G1
NRP_19	3	62	0,5	7,5	55	3,16	1,61	0,95	0,28	0,08	21	38	-	3	1	IIIB	G1	Nebivolon	CAPE **	G1	G1
RP_1	2	75	1,12	6,8	86	2,57	1,03	0,29	0,14	0,01	-	-	-	3	1	IIIB	G2	Ticlopidine;PE;IDP; ENP	CAPE **	G0	G3
RP_2	1	74	0,94	7,8	133	3,15	1,38	0,29	0,06	0,01	17	17	446	3	1	IIIB	G1	-	CAPE **	G0	G0
RP_3	2	50	0,8	7,4	98	4,72	2,46	0,62	0,22	0,04	33	39	831	2	1	IIIA	G2	-	CAPE **	G0	G1
RP_5	2	64	0,9	7,2	68	3,29	2,16	0,29	0,14	0,01	20	39	357	2	0-1	IIIA	G2	LPZ	CAPE *+ OXA§	G1	G2
RP_8	2	65	0,78	7,2	99	4,56	1,16	0,48	0,06	0,02	19	18	559	3	1	IIIB		ENP+ HCTZ; Dox	CAPE *+ OXA§	G0	G2
RP_9	1	41	0,69	7,7	78	3,92	1,69	0,44	0,14	0,02	16	24	375	3	2	IIIB	G2	Rosuvastatin	CAPE *+ OXA§	G1	G2
RP_12	1	66	0,56	7,1	83	6,42	2,55	0,48	0,19	0,06	25	47	466	3	1	IIIB	G1	Quinapril + HCTZ	CAPE **	G0	G2
RP_17	2	53		6,9	98	-	-	-	-	-	29	20	548	3	0	II	G2	Pioglitazone; ENP	CAPE **	G1	G2

Table S2: MS/MS fragmentation functions performed for the detection of the subclasses of phospholipids investigated.

Phospholipid class (Parent)	MS/MS function	Collision Energy	Acquisition time
PG	Neutral loss of 172 m/z	15	2-15 min
PE	Neutral loss of 141 m/z	15	7-14 min
PS	Neutral loss of 185 m/z	20	10-20min
PC, SM	Parent scan of 184 m/z	25	10-21 min

Table S3: Reproducibility of the Retention Time (RT) and peaks area evaluated for each standard lipid analysed.

ANALYTE	**RT (min)**		**PEAKS AREA**		**[M+H$^+$] (m/z)**	
	AVERAGE	CV%	AVERAGE	CV%	AVERAGE	CV%
PC (14:0/14:0)	13.39	1.07	5.09E+08	11.06	678.6	0.05
SM (d18:1/16:0)	14.97	0.33	7.95E+07	10.09	703.9	0
LPC (16:0/0:0)	16.5	0.37	7.91E+07	13.58	468.4	0.01
LSM (d18:1/0:0)	18.99	0.19	5.68E+07	13.11	465.5	0.02
PE (14:0/14:0)	10.32	0.3	1.43E+07	10.89	636.9	0.01
LPE (16:0/0:0)	12.32	0.29	4.39E+06	10.49	454.7	0.01
PG (16:0/16:0)	2.81	1.84	6.92E+06	19.13	724	0
PS (18:0/18:0)	12.47	3.54	4.20E+06	14.79	789.3	0.09

Table S4: MRM functions refer to LC-MS/MS targeted experiment created for each lipid of interest. In the table are listed the transitions related to each lipid signal belonging to the four compound classes. Internal standard was reported in bold.

MRM Function	Time window (min)	Compound Class	Transitions	Cone (V)	Collision Energy (eV)
1	1.0-7.0	PG	337.0>165.0 719.6>547.6 877.7>705.7 913.5>741.5 798.5>626.5 501.4>329.4	35	15
2	7.0-12.0	PE	812.9>671.9 478.6>337.6 723.0>582.0 528.6>387.6 502.7>361.7 532.4>391.4 750.0>609.0	35	15
3	8.0-16.0	PS	782.5>597.5 741.5>556.5 879.5>694.5 840.4>655.4 716.6>531.6 879.5>694.5	35	20
4	12.0-25.0	PC/SM	727.8>184.0 992.4>184.0 496.2>184.0 480.4>184.0 787.5>184.0 842.9>184.0 ***465.6>184.0*** 842.0>184.0	35	25

Table S5: Number of variables taken into consideration for each phospholipids class analysed and the parameters of the PLS-DA models obtained.

Class	Obsevations	R2X	Q2(cum)	N. of components
PC	271	0.324	0.652	4
PE	408	0.312	0.74	4
PG	378	0.263	0.668	4
PS	706	0.293	0.629	4

Table S6: The table lists identification and the clinical parameter by which the validated lipid has a significant correlation.

Retention Time (min)	Mass (m/z) $[M+H^+]$	Common name	AUC	Clinical correlation
14.79	727.86	SM(d18:2/18:1)	0.77	Monocytes
16.14	496.22	Lyso PC(16:0/0:0)	0.92	Eosinophils
15.72	480.42	Lyso PC(15:1(9Z)/0:0)	0.9	
13.86	842.90	PC(20:0/20:2) PC(20:1/20:1) PC(18:0/22:2) PC(18:1/22:1) PC(18:2/22:0) PC(16:1/24:1)	0.93	
11.08	528.61	Lyso PE(22:5/0:0)	0.78	Lactic dehydrogenase

Bibliography

1. Genovesi D, Filippone A, Ausili Cefaro G, Trignani M, Vinciguerra A, Augurio A, Di Tommaso M, Borzillo V, Sabatino F, Innocenti P, Liberatore E, Colecchia G, Tartaro A, Cotroneo AR. Diffusion-weighted magnetic resonance for prediction of response after neoadjuvant chemoradiation therapy for locally advanced rectal cancer: Preliminary results of a monoinstitutional prospective study. *European journal of surgical oncology*

: the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology 2013;39:1071-1078.
2. Mandard AM, Dalibard F, Mandard JC, Marnay J, Henry-Amar M, Petiot JF, Roussel A, Jacob JH, Segol P, Samama G, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer* 1994;73:2680-2686.
3. Xiong Y, Zhao YY, Goruk S, Oilund K, Field CJ, Jacobs RL, Curtis JM. Validation of an lc-ms/ms method for the quantification of choline-related compounds and phospholipids in foods and tissues. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 2012;911:170-179.
4. Xia J, Psychogios N, Young N, Wishart DS. Metaboanalyst: A web server for metabolomic data analysis and interpretation. *Nucleic acids research* 2009;37:W652-660.