Supplementary Information

Metabolomic Profiling Predicts outcome of Rituximab therapy in Rheumatoid Arthritis.

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Methods

Materials

LC-MS grade methanol, acetonitrile, isopropanol, chloroform, formic acid, and ammonium acetate were purchased from Thermo Fisher Scientific (San Jose, CA). Mass spectrometry calibration mixtures were also purchased from Thermo Fisher Scientific (Thermo Scientific Pierce LTQ Velos ESI Positive and Negative Ion calibration solutions). The following internal standards for mass spectrometry, 1,2-dipalmitoyl-d62-sn-glycero-3-phosphoethanolamine (16:0 PE-d62), 1,2-dimyristoyl-d54-sn-glycero-2-[phospho-L-serine] sodium salt (14:0 PS-d54), 1,2-dilauroyl-sn-glycero-2-phosphocholine (12:0 PC), 1,2-dioleoyl-sn-glycero-3-phosphoinositol ammonium salt (18:1 PI), and Ceramide/Sphingoid Internal Standard Mixture I were acquired from Avanti Polar Lipids Inc. (Alabaster, Alabama). In addition, heptadecanoic-17,17,17-d3 acid was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Deuterated 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TMSP-d4) was used as an internal standard for NMR experiments and was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). D_2O for NMR experiments was sourced from Acros Organics (Fair Lawn, NJ).

Sample preparation for metabolomics analysis

A total of 43 samples were prepared and analyzed using NMR and UPLC-MS/MS analytical platforms[15 24-28]. Frozen sera were obtained from the Division of Rheumatology, Allergy, and Immunology at UC San Diego School of Medicine (San Diego, CA). Lipid and protein fractions were removed via ultrafiltration (Nanosep 3K OMEGA, Pall Corporation, Ann Arbor, MI) at 4°C.

The filtered biofluid was used for NMR analysis. An aliquot of 160 μL of filtered serum was mixed with 20 μL D_2O and 20 μL of phosphate buffer (100 mM final concentration) containing TMSP-d4 (0.1 mM final concentration) and sodium azide (0.05% (w/v) final concentration). The prepared samples were centrifuged to remove any remaining particulates and a 180 μL aliquot was transferred to a 3 mm NMR tube (Norell, Landisville, NJ) prior to acquisition. NMR spectra were acquired with a 16.4 T (700 MHz) Bruker Avance spectrometer (Bruker BioSpin Corp., Billerica, MA) equipped with a 5 mm TCI cryogenically cooled probe and autosampler at 30°C.

The apolar fraction was recovered from the filter by two successive washes with 250 μL of 0.9% saline solution and then spiked with several stable isotope labeled internal standards. Recovered lipid fractions were isolated by extraction with 1 mL chloroform, 500 μL methanol, and 500 μL saline containing recovered metabolites [15]. Individual samples were vortexed immediately to ensure protein inactivation. Subsequently, samples were agitated at 2500 rpm using a platform shaker (Heidolph, Elk Grove Village, IL) for 10 minutes. Samples were then phase separated by centrifugation at 4750 rpm for 20 minutes at 4°C. A 200 μL aliquot of recovered lipid containing chloroform phase was transferred into a glass tube. The aliquots were dried using a CentriVap Concentrator (Labconco, Kansas City, MO) at -4°C. Dried samples were resuspended in 200 μL of 65:30:5 acetonitrile:isopropanol:water and transferred to glass liquid chromatography (LC) autosampler vials (Supelco, Bellefonte, PA). UPLC-MS analysis was performed on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer equipped with an Accela 1250 pump and autosampler (Thermo Scientific, Waltham, MA).

NMR analysis and data processing

NMR spectra were acquired with a 16.4 T (700 MHz) Bruker Avance spectrometer (Bruker BioSpin Corp., Billerica, MA) equipped with a 5 mm TCI cryogenically cooled probe and autosampler at

30°C. Each sample was allowed to equilibrate for 5 minutes in the probe prior to acquisition. One dimensional ^{1}H-NMR pulse sequence with excitation sculpting water suppression was used for data acquisition [29]. Following acquisition, spectra were processed using NMRlab and MetaboLab [30] in the MATLAB programming environment (MathWorks Inc., Natick, MA). Metabolites concentrations were normalized to the TMSP-d4. Post-processing for statistical analysis included scaling, alignment, exclusion of regions containing water and TMSP peaks, and generalized log transformation [31]. Metabolite assignment and quantification was performed using Chenomx NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada), the Birmingham Metabolite Library [32], and the Human Metabolome Database [33].

MS analysis and data processing

UPLC-MS analysis was performed on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer equipped with an Accela 1250 pump and autosampler (Thermo Scientific, Waltham, MA). Lipids were separated on a Kinetex 2.6 μm C-18 100 Å column (Phenomenex, Torrance, CA). An 80:20 solution of water and acetonitrile with 10 mM ammonium acetate and 0.05% (w/v) formic acid was used for mobile phase A. For mobile phase B, a solution of 90:9:1 isopropanol:acetonitrile:water with 10 mM ammonium acetate and 0.5% (w/v) formic acid was prepared. Separation was achieved by means of a gradient beginning with 90% mobile phase A, decreasing to 5% A in 20 minutes, holding at 5% for 7 minutes, and then increasing to 90% A in 8 minutes to re-equilibrate the column. The total run time was 35 minutes with a flow rate of 0.3 mL/min. Eluent entered the ion trap via an electrospray ionization (ESI) source. For negative ionization mode, the spray voltage was set to 3.0 kV, whereas the capillary temperature was set to 300°C. The sheath gas flow was 45 units and the auxiliary gas was 11 units. In the positive ionization mode acquisitions, spray voltage and capillary

temperature were adjusted to 2.5 kV and 275°C respectively. Solvent blanks and pooled quality controls were injected periodically to monitor column carry over and instrument stability.

Data acquisition was performed using the Thermo Scientific software package Xcalibur and then further imported into Sieve Software 2.1 (Thermo Scientific) for processing. The features obtained in Sieve were then imported and analyzed in MATLAB. Spectra deconvolution were processed according to mass accuracy and retention time. Metabolite assignment was done at a 5 ppm mass accuracy range by interrogation of several databases including KEGG [34], LIPID MAPS [35] Human Metabolome Database (HMDB) [33], Madison Metabolomics Consortium Database (MMCD) [36], and Metlin [37], in part using MetaboSearch [38], a software tool developed for untargeted MS-based metabolomics. Intensities were normalized to internal standards that were present in all samples. For negative ionization mode, heptadecanoic-17,17,17-d3 was used. (2S,3R)-2-aminoheptadecane-1,3-diol (Sphinganine C17) was used for normalization of intensities generated in positive ionization mode. Following normalization, metabolites identified in each mode were merged to generate a composite list of metabolites prior to statistical analysis in MATLAB. Where accurate masses and retention times were associated with multiple lipid classes, MS/MS analysis was performed to fragment the molecules of interest to improve assignment confidence.

Pathway analysis

MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used identify pathways of significant interest involving polar metabolites[39] [40]. In addition, VANTED (http://vanted.ipk-gatersleben.de/) software was used for lipid pathway analysis of statistically significant lipid classes [41]. The upregulation or downregulation of individual lipid species was evaluated by calculating the fold change between responders and non-responders. A global test algorithm was applied to determine the overall significance of each pathway between the two groups[42]. Relative abundances are reported

as colors to indicate the direction of statistically significant differences between groups. Polar and apolar pathways were manually integrated to provide a more comprehensive picture of the overall metabolic dysregulation found in rheumatoid arthritis patients who either did or did not respond to rituximab therapy.

Supplementary Table 1. Baseline characteristics of rheumatoid arthritis patients, by response to rituximab at 6 months

Clinical Parameter	**Responders (n = 14)**	**Non-responders (n = 9)**
Age (years)	55.6±12.2	54.5±15.3
Female (%)	85	77.7
DAS28	6.5±0.8	6.35±1.36
ESR (mm/h)	44.2±26.4	48.8±37.2
HAQ	1.75±0.73	1.95±0.7
Pain	60.5±24.3	68.8±22.7
Swollen Joints	25.5±14.3	22.1±18.6
Tender Joints	26.8±14.1	25.5±16.7
Swollen Joints (28)	15.4±8.3	14±8.9
Tender Joints (28)	16.8±8.5	14±7.9
CCP positive (U)	188.5±252.2	76.6±56.2
$CD19^{+}$ cells (%)	0.13±0.15	0.1±0.04
ACR response	57.2±20.9	10±15.8
Baseline MTX dose (mg/wk)	15.3±5.5	16.3±5.3
Baseline prednisone dose (mg/day)	5±4.3	7±2.5
Previous anti-TNF therapy (%)	64.3	77.7

Supplementary Table 2. Polar metabolites identified and quantified by 1D [1]H-NMR before and after treatment with rituximab. Reference values are from HMDB [33] and were collected via NMR, unless otherwise noted. [1]GC/MS [2]HPLC [3]HPLC-Fluoroescence [4]Ion-exchange chromatography [5]DFI/MS/MS [6]Unknown. ND: No data available.

		Before				After			
		Responders		Non-Responder		Responders		Non-Responder	
Metabolite	Normal Range (uM)	Mean (uM)	St. Dev.	Mean (uM)	St. Dev.	Mean (uM)	St. Dev.	Mean (uM)	St. Dev.
2-Hydroxyvalerate	ND	4.74	1.92	11.15	9.49	49.41	12.20	60.12	11.87
3-Hydroxybutyrate	76.9±66.3	79.57	64.49	141.58	101.46	33.56	12.80	43.16	23.85
Acetoacetate	40.6±36.5	10.11	3.87	16.27	9.73	5.75	1.48	7.00	2.85
Acetone	54.4±29.6	15.03	7.47	18.88	12.94	16.55	6.10	16.40	4.80
Alanine	427.2±84.4	360.34	99.62	404.54	155.44	388.91	58.24	405.61	158.20
Asparagine	82.4±7.3	25.83	8.31	29.02	5.55	60.27	11.62	61.05	9.32
Aspartate	20.9±6.1	70.29	27.76	86.04	46.60	51.94	14.55	67.56	16.64
Azelate	27 (0 - 58)[1]	87.03	64.28	84.60	58.48	128.83	89.86	115.62	74.98
β-Alanine	3.8±2.9[2]	2.54	0.60	3.03	1.49	11.56	1.99	13.61	4.20
Betaine	72±22.4	52.94	16.55	63.59	18.15	50.07	10.94	54.63	10.73
Carnitine	45.7±11.6	25.50	6.58	28.05	11.30	37.92	7.60	36.32	4.19
Choline	14.5±5.3	8.07	2.42	11.62	4.93	13.70	3.20	13.40	3.45
Citrate	114.2±27	115.87	28.67	129.33	51.96	113.79	19.17	127.02	32.18
Creatine	37.6±28.3	27.25	15.73	27.14	10.47	14.77	6.58	16.75	8.85
Creatinine	86.6±18.8	54.18	12.62	67.49	22.70	58.11	9.18	65.59	11.56
Dimethylamine	48.35±7.32[2]	1.60	0.69	1.86	0.49	6.94	3.67	5.51	1.10
Dimethylsulfone	8.8±7.3	12.98	26.99	2.40	0.73	23.24	57.14	7.36	0.70
Formate	32.8±13.3	19.93	6.13	23.07	9.64	22.78	4.94	23.86	4.87
Fumarate	1.5 (0 - 4)[1]	0.88	0.45	1.33	1.25	0.40	0.57	0.54	1.00
Glucose	4971.3±372.8	4060.62	2338.39	3878.75	1549.05	4609.38	2155.66	5263.56	2549.87
Glutamate	97.4±13.2	158.66	45.85	215.36	98.52	39.60	9.98	42.85	14.07
Glutamine	510.4±118.2	513.77	136.99	535.51	156.40	376.05	57.24	396.41	97.03
Glycerol	431.6±100.4	73.64	18.02	97.32	45.11	104.56	95.48	94.08	39.17
Glycine	325.4±126.8	241.62	75.73	313.43	71.69	266.35	55.89	298.21	46.56
Hypoxanthine	34.2±10.3	13.42	5.10	21.15	21.03	11.37	4.90	13.33	4.75
Isoleucine	60.7±18.6	57.16	21.40	61.55	12.57	64.19	16.29	59.27	19.38

Lactate	1489.4±371.2	3600.75	3385.26	4101.73	2338.40	2172.43	675.38	3316.75	1387.23
Leucine	98.7±11.5	140.86	44.16	165.52	36.06	138.42	30.67	135.97	27.56
Lysine	178.6±58.2	137.43	34.31	133.63	38.50	125.45	21.36	126.06	29.17
Methionine	29.8±6.3	19.37	5.36	21.19	7.93	35.32	6.19	35.76	5.01
Methylmalonate	0.187±0.084[3]	20.07	15.16	27.40	12.46	11.67	2.90	13.38	6.14
Methylsuccinate	ND	12.70	3.24	13.73	3.59	4.17	1.15	4.03	0.67
O-acetylcarnitine	5.476±2.147[5]	3.92	1.14	4.79	1.97	3.50	0.78	4.14	0.92
Ornithine	66.9±15.3	46.38	17.91	58.77	33.95	52.20	11.01	56.31	23.86
Oxoisocaproate	28 (0 - 58)[1]	8.76	2.67	8.92	3.47	13.07	2.78	14.02	2.38
Pantothenate	4.91±0.38[6]	2.32	0.56	2.85	1.04	19.93	4.97	24.41	5.01
Phenylalanine	78.1±20.5	60.88	20.01	83.00	21.66	60.14	14.63	58.79	11.70
Propyleneglycol	22.3±3.3	176.07	129.63	197.27	133.84	205.90	139.91	173.06	100.80
Pyruvate	34.5±25.2	60.52	28.81	83.40	50.28	46.13	17.31	78.89	44.55
Serine	159.8±26.6	153.98	35.48	182.14	51.03	138.51	20.37	152.02	37.89
Succinate	23.5±16.0[1]	8.01	3.83	12.88	6.11	10.82	2.02	14.46	1.44
Taurine	55±13[4]	238.77	54.21	279.25	54.72	120.23	13.56	140.44	21.89
Threonine	127.7±41	202.79	219.76	514.50	513.37	118.09	14.03	119.12	22.71
Trimethylamine	0.418±0.124[1]	0.85	0.50	1.22	0.76	1.84	0.98	2.18	1.28
Tyrosine	54.5±9.7	56.67	16.66	74.21	22.88	66.58	18.81	71.57	16.24
Urea	6074.6±2154.2	5716.72	2124.47	6101.60	2964.36	13458.43	4387.49	12896.35	4362.01
Valine	212.3±61.3	205.42	68.10	223.46	45.24	240.52	40.65	218.62	30.61
Xanthine	1.27±0.78[2]	8.01	3.01	6.06	3.56	7.08	1.83	7.22	2.69

Supplementary Table 3. Lipid species identified as statistically significant between groups prior to treatment with rituximab. For simplicity, only one arrangement of each fatty acid combination is given. Alternative fatty acid combinations are indicated where appropriate. RT: retention time (minutes), PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, O: alkyl ether, P: alkenyl ether

Lipid	Mass	RT	p-value
PS(O-20:0/18:0)/(O-16:0/22:0)	804.6132	19.64	0.0036
GlcCer(d15:1/18:0)	686.5575	20.55	0.0064
PE(O-20:0/22:6)	804.5916	21.62	0.0116
PS(22:6/20:4)	854.4976	17.51	0.0174
PC(O-17:0/20:4)	288.2899	19.69	0.0255
PC(O-16:1/2:0)/(P-16:0/2:0)/(18:1/0:0)	782.6051	13.53	0.0270
PI(P-16:0/17:2)	522.3551	19.34	0.0294
PG(17:0/21:0)/(22:0/16:0)/(19:0/19:0)/(20:0/18:0)	803.5085	19.63	0.0312
TG(17:2/17:2/18:3)/(16:1/18:3/18:3)	805.5968	18.07	0.0374
N-stearoyl glycine/N-palmitoyl GABA/Cer(d18:1/2:0)	849.6959	19.90	0.0377
PC(15:0/18:2)/(13:0/20:2)/(14:1/19:1)/(15:1/18:1)/(16:0/17:2)	342.3007	19.34	0.0384
PI(16:1/22:2)/(18:1/20:2)/(18:2/20:1)/(18:3/20:0)	744.5541	18.44	0.0450
PS(17:1/22:2)/(17:2/22:1)/(18:3/21:0)/(19:0/20:3)	887.5659	10.83	0.0475
PS(O-16:0/21:0)/(O-18:0/19:0)/(O-20:0/17:0)	826.5599	22.44	0.0480
PI(14:1/22:4)/(18:1/18:4)/(18:2/18:3)	790.5960	19.67	0.0499

Supplementary Table 4. Lipid species identified as statistically significant between groups following treatment with rituximab. For simplicity, only one arrangement of each fatty acid combination is given. Alternative fatty acid combinations are indicated where appropriate. RT: retention time (minutes), PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, O: alkyl ether, P: alkenyl ether

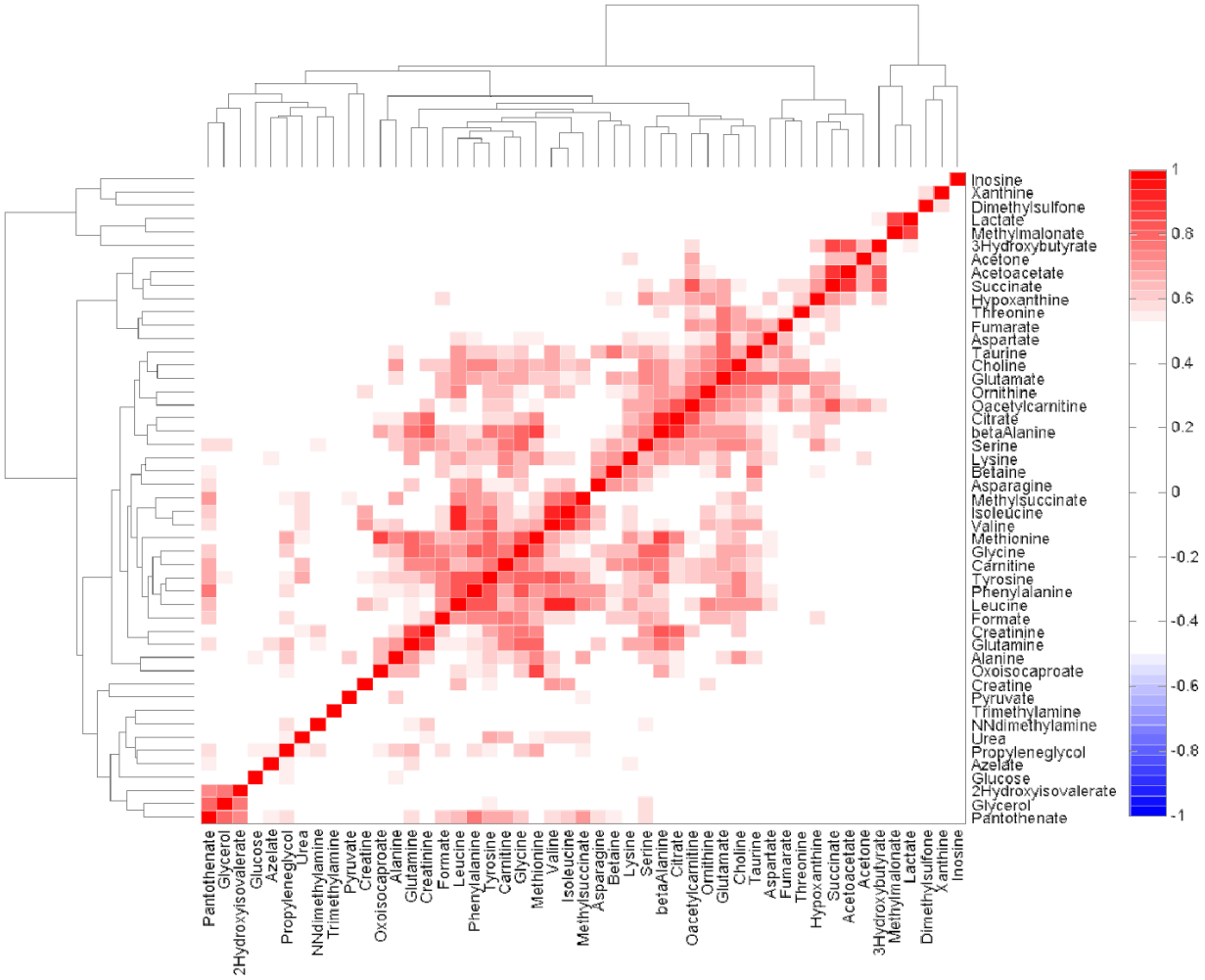
Lipid	Mass	RT	p-value
22:1-Glc-cholesterol/20:0-Glc-Stigmasterol/20:1-Glc-Sitosterol	869.7216	19.64	0.0002
PS(18:1/22:4)/(18:3/22:2)	838.5604	20.55	0.0016
Cer(d18:0/24:0)/(d20:0/22:0)	652.6608	21.62	0.0024
PS(O-16:0/22:4)/(O-20:0/18:4)/(O-18:0/20:4)/(P-18:0/20:3)	798.5649	17.51	0.0037
PC(15:0/18:1)/(16:0/17:1)/(13:0/20:1)/(14:0/19:1)/(14:1/19:0)/(15:0/18:1)/(15:1/18:0)	744.5542	19.69	0.0045
PS(18:0/20:4)/(18:1/20:3)/(18:2/20:2)/(18:3/20:1)	272.2675	13.53	0.0075
SM(d18:2/24:1)	810.5279	19.34	0.0080
PS(O-16:0/20:1)/(O-18:0/18:1)/(O-20:0/16:1)/(P-16:0/20:0)/(P-18:0/18:0)/(P-20:0/16:0)	811.6687	19.63	0.0123
PC(O-17:0/22:0)/PC(O-18:0/21:0)/PC(O-20:0/19:0)/PE(O-20:0/22:0)	776.5810	18.07	0.0136
PC(15:0/18:2)/(13:0/20:2)/(14:1/19:1)/(15:1/18:1)/(16:0/17:2)	818.6986	19.90	0.0165
GlcCer(d18:1/16:0)/GlcCer(d14:1/20:0)/GlcCer(d16:1/18:0)/GalCer(d18:1/16:0)	742.5403	19.34	0.0165
PG(O-20:0/22:1)(P-20:0/22:0)	700.5726	18.44	0.0192
TG(17:1/18:4/18:4)/(13:0/18:3/22:6)	288.2899	10.83	0.0196
TG(17:2/17:2/18:3)/(16:1/18:3/18:3)	847.6780	22.44	0.0209
PE(16:0/0:0)	857.6670	19.67	0.0211
PC(13:0/18:2)/(14:0/17:2)/(14:1/17:1)/(15:1/16:1)	849.6959	22.70	0.0236
PS(O-16:0/20:2)/(O-18:0/18:2)/(P-16:0/20:1)/(P-20:0/16:1)/(P-18:0/18:1)	454.2934	12.30	0.0238
PC(13:0/18:2)/(14:0/17:2)/(14:1/17:1)/(15:1/16:1)	716.5234	18.64	0.0240
SM(d18:2/22:1)	774.5641	17.53	0.0268
TG(12:0/12:0/15:1)/(12:0/13:0/14:1)	714.5086	18.67	0.0314
PC(13:0/20:4)/(15:0/18:4)/(15:1/18:3)/(15:1/18:3)	783.6384	19.05	0.0331
PS(O-16:0/22:2)/(O-18:0/20:2)/(O-20:0/18:2)/(P-16:0/22:1)/(P-18:0/20:1)	679.5864	22.05	0.0334
N-stearoyl serine	740.5214	18.50	0.0357
PC(13:0/20:4)/(15:0/18:4)/(15:1/18:3)	802.5964	18.30	0.0377
PA(O-16:0/18:3)/(P-16:0/18:2)	372.3112	10.03	0.0383
PC(O-16:0/18:1)/(O-18:0/16:1)/(O-20:0/14:1)/(P-16:0/18:0)/(P-20:0/14:0)	738.5081	18.50	0.0408
PG(O-20:0/20:0)/(O-18:0/22:0)	657.4860	20.17	0.0411
Axillarenic acid/Tetracosanedioic acid	746.6049	19.36	0.0411
beta-hydroarchaetidylglycerol	821.6632	22.34	0.0426
18:3 Campesteryl ester/ergosteryl oleate	399.3471	17.20	0.0428
PC(O-16:0/22:2)/(O-18:0/20:2)/(O-20:0/18:2)/(P-16:0/22:1)/(P-18:0/20:1)	823.6788	22.63	0.0443
PS(15:0/22:2)/(15:1/22:1)/(17:0/20:2)/(17:1/20:1)/(17:2/20:0)	659.5770	21.04	0.0443
PA(22:1/22:2)	800.6517	20.11	0.0451
Anandamide (20:2, n-6)	800.5450	18.63	0.0462
PG(21:0/22:1)	811.6206	22.26	0.0472
PC(18:0/18:1)	352.3213	10.67	0.0487
PA(12:0/21:0)/(14:0/19:0)/(17:0/16:0)/(18:0/15:0)/(20:0/13:0)	875.6725	20.04	0.0497

Supplementary Table 5. Abundance of glycerophospholipid classes in responders relative to non-responders before and after treatment with rituximab. Arrows indicates abundance relative to non-responders. N/S: not significant, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, O: alkyl ether, P: alkenyl ether.

Before Treatment	Responders	After Treatment	Responders
PA	**N/S**	**PA**	↑
PC	↑	**PC**	↑
O/P-PC	↓	**O/P-PC**	↓
PE	↓	**PE**	↓
O/P-PE	↓	**O/P-PE**	**N/S**
PG	↓	**PG**	↓
O/P-PG	**N/S**	**O/P-PG**	↑
PI	↑	**PI**	**N/S**
O/P-PI	↓	**O/P-PI**	↓
PS	↓	**PS**	↑
O/P-PS	↓	**O/P-PS**	↓

Supplementary Table 6. PLSDA Model statistics.

	Before		**After**	
Preprocessing	Autoscale		Autoscale	
Number of LVs	2		2	
Cross Validation	Venetian Blinds 10 splits, 1 sample/split		Venetian Blinds 4 splits, 1 sample/split	
Modeled Class	1	2	1	2
Sensitivity (Cal)	1.000	1.000	1.000	1.000
Specificity (Cal)	1.000	1.000	1.000	1.000
Sensitivity (CV)	0.857	0.111		0.429
Specificity (CV)	0.111	0.857	0.429	0.538
Class Error (Cal)	0	0	0	0
Class Error (CV)	0.519	0.519	0.516	0.516
RMSEC	0.150	0.150	0.085	0.085
RMSECV	790.814	790.814	0.692	0.692
Bias	-1.110e-16	5.551e-17	1.110e-16	0
CV Bias	168.615	-168.615	0.007	-0.007
R^2 (Cal)	0.905	0.905	0.968	0.968
R^2 (CV)	0.027	0.027	0.001	0.001



Supplementary Figure S1. Heat map and hierarchical cluster analysis indicate positive relationships between polar metabolites identified by ^{1}H-NMR in sera from RA patients before treatment with rituximab. Pearson's correlation coefficients for each metabolite and hierarchical clustering with Euclidean distance metric.