Supplementary Information.

Integrative biochemical, proteomics, and metabolomics cerebrospinal fluid biomarkers predict clinical conversion to multiple sclerosis.

Running head: Integrative biomarkers predict conversion to MS

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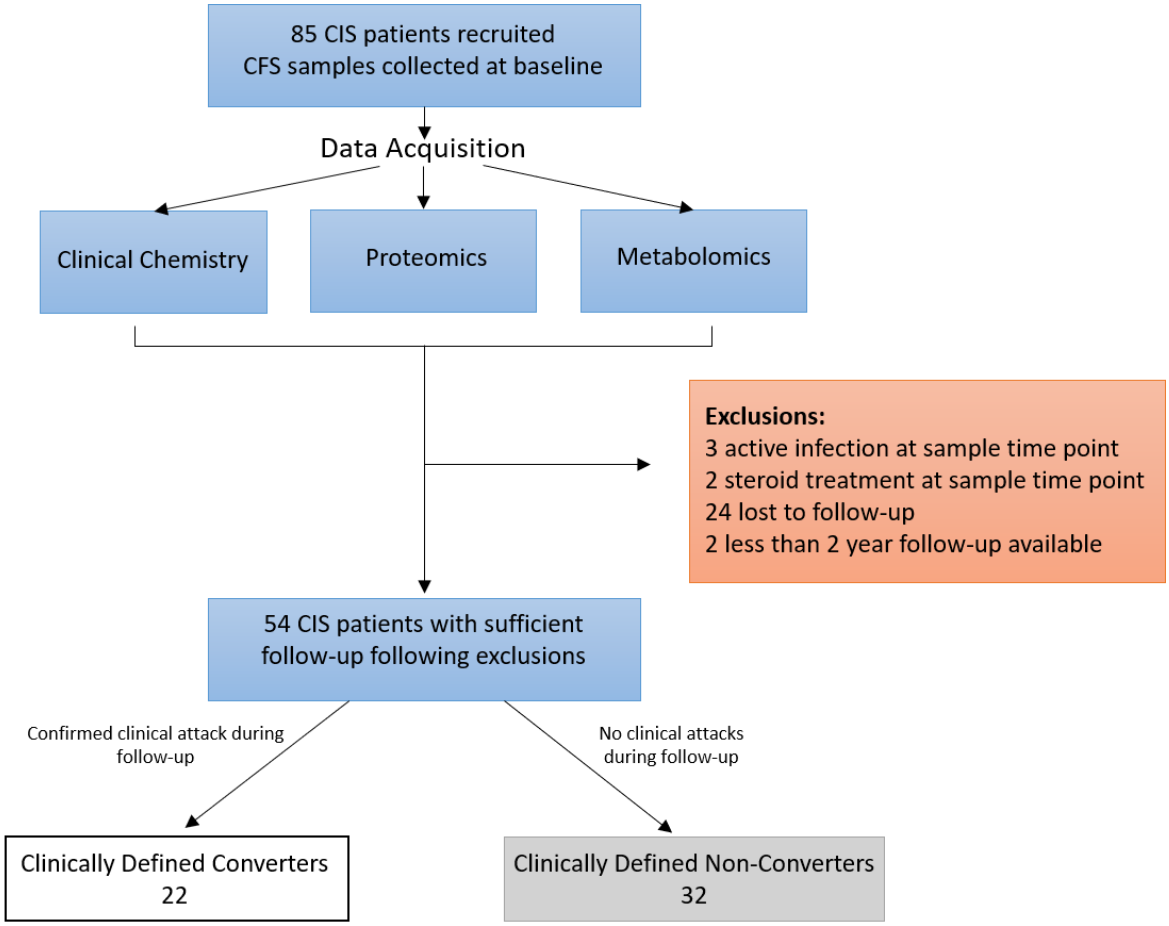
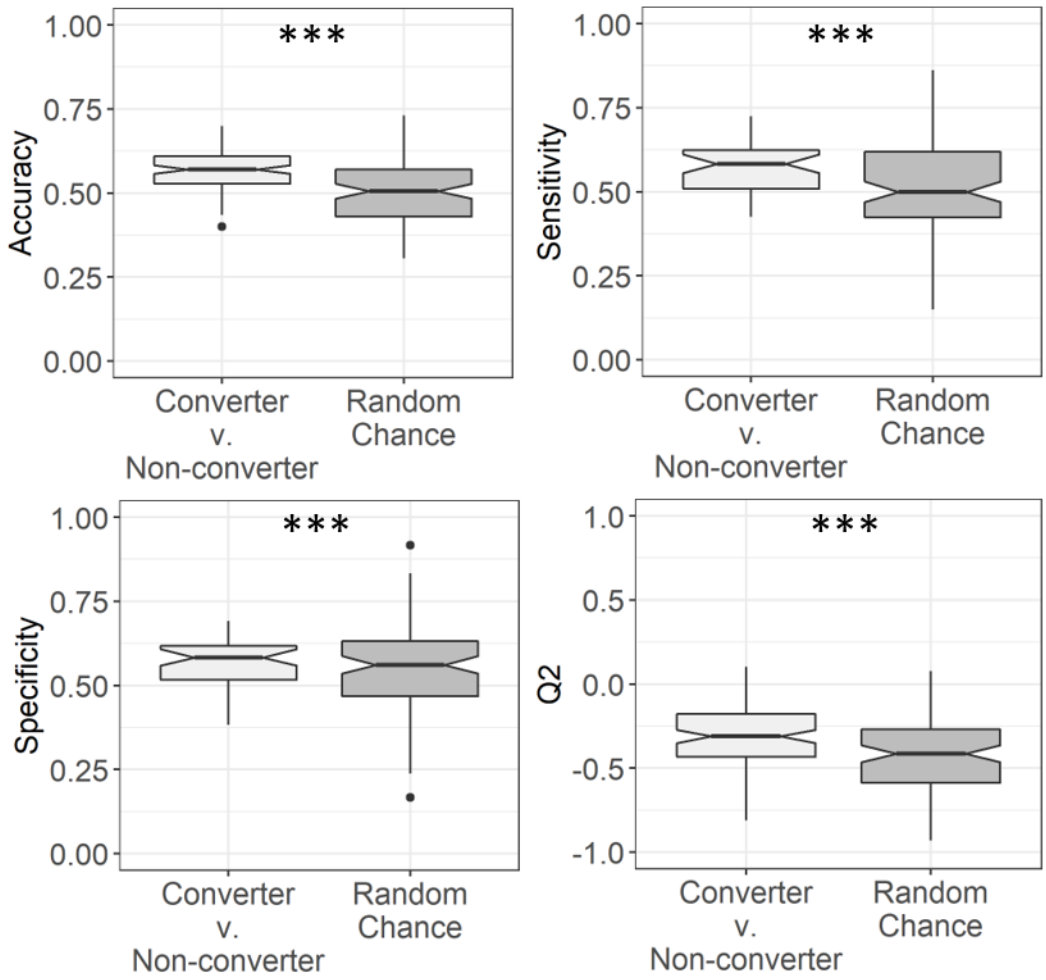
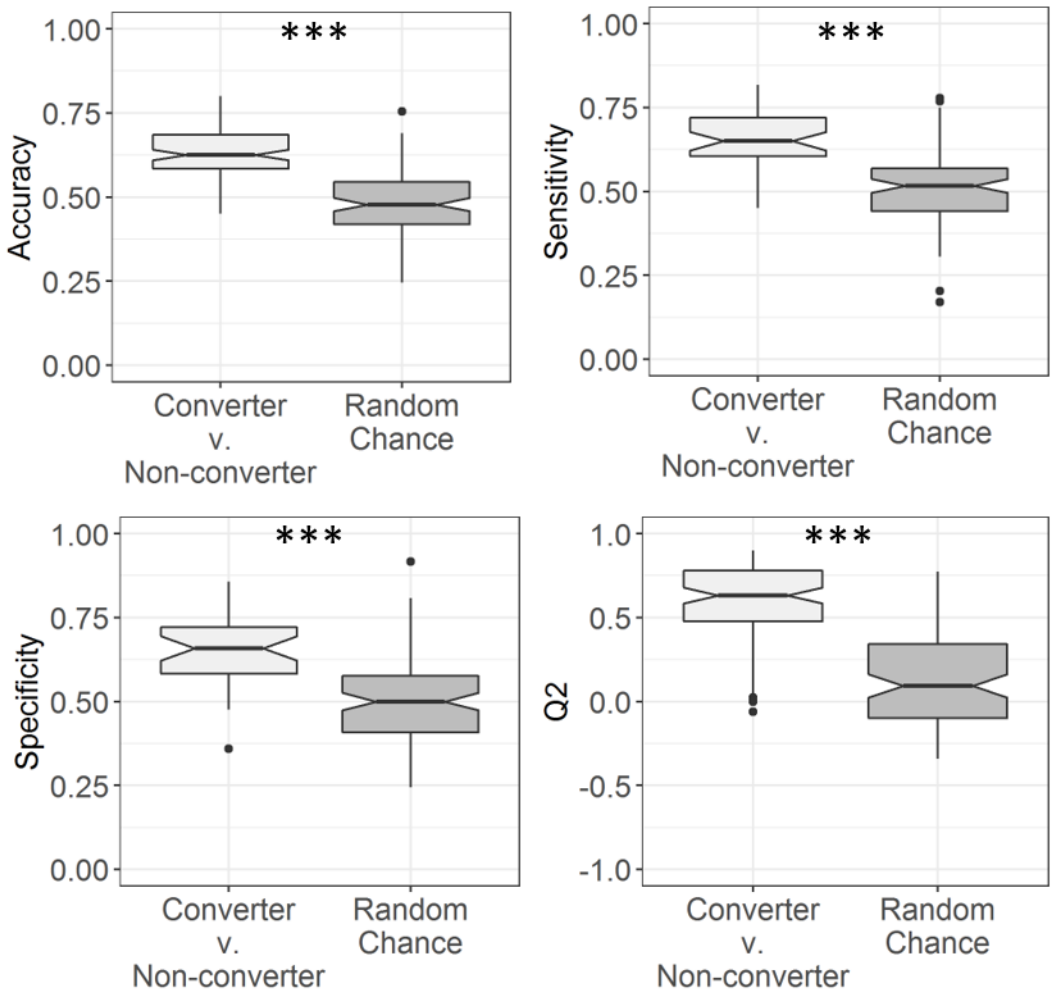


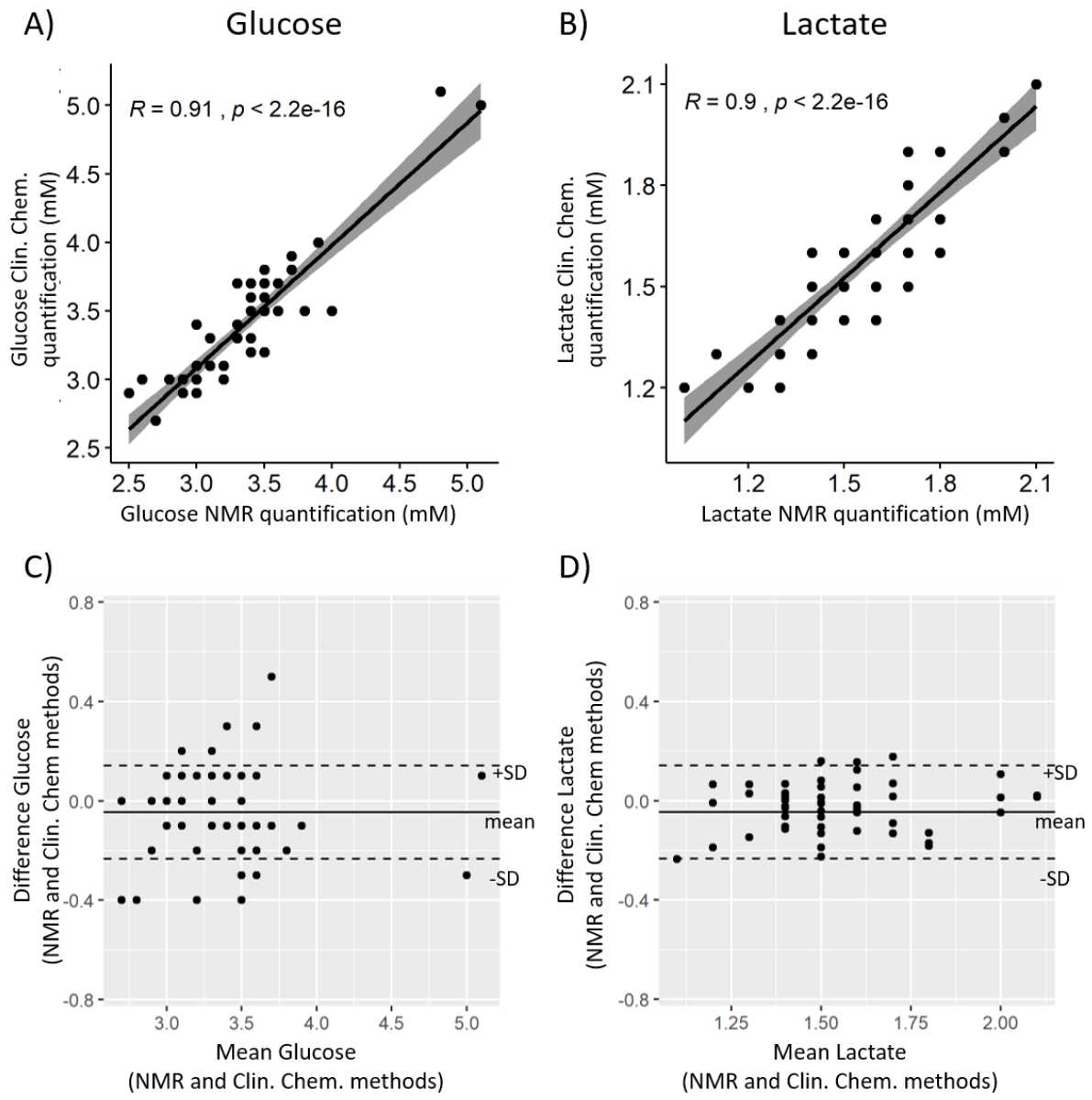
Supplementary Figure 1. Flow diagram illustrating numbers of individuals included in the study and reasons for exclusion. 85 patients were recruited at CIS onset, CSF samples were collected at baseline and clinical chemistry, proteomics, and metabolomics data was acquired on all samples. 26 of the patients either had no follow-up data or less than 2 years of follow-up and thus were excluded from the analysis. Three patients were confirmed to have active infections and two were receiving steroid treatment at the time of CSF sampling and were excluded from the analysis. Thus, a total of 54 patient samples were confirmed to be eligible analysis and were stratified into clinically defined converter and non-converter groups using the follow-up data.



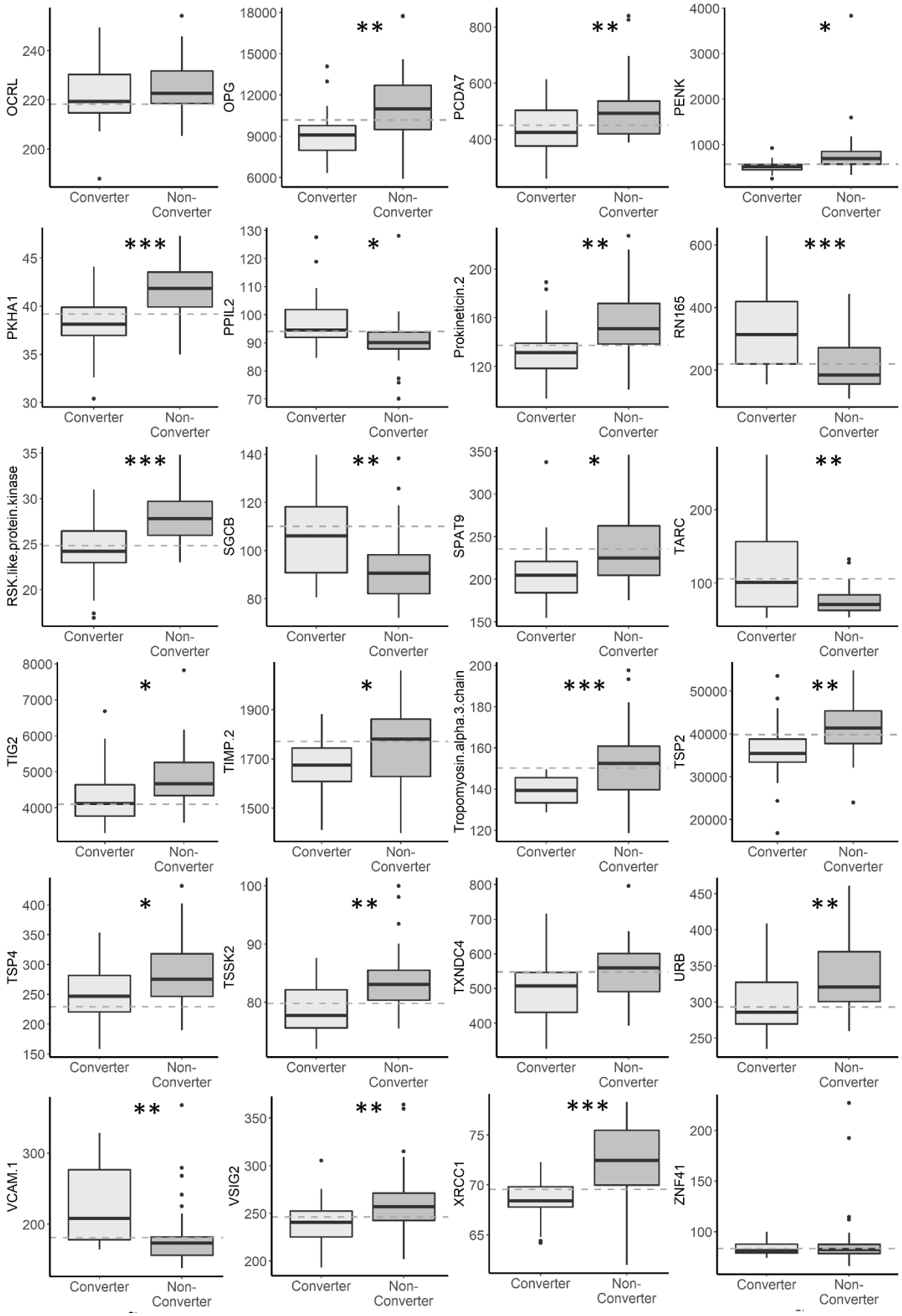
Supplementary Figure 2. Validating metabolomics OPLS-DA models on independent test data. 10-fold cross-validation with repetition reveals significantly increased accuracy, sensitivity, specificity and Q2 on independent test data (excluded when training the model) relative to the null distribution produced by permutation testing (the performance expected by random chance alone). Two-sample Kolmogorov-Smirnov p-values < 0.001 are represented by ***.



Supplementary Figure 3. Validating proteomics OPLS-DA models on independent test data. 10-fold cross-validation with repetition reveals significantly increased accuracy, sensitivity, specificity and Q2 on independent test data (excluded when training the model) relative to the null distribution produced by permutation testing (the performance expected by random chance alone). Two-sample Kolmogorov-Smirnov p-values < 0.001 are represented by ***.



Supplementary Figure 4. Agreement between metabolite concentrations determined by NMR metabolomics and standard laboratory chemistry methods. Significant Pearson linear correlations were observed for (A) glucose and (B) lactate concentrations. (C-D) Bland-Altman plots illustrate excellent agreement between the two measurement methods.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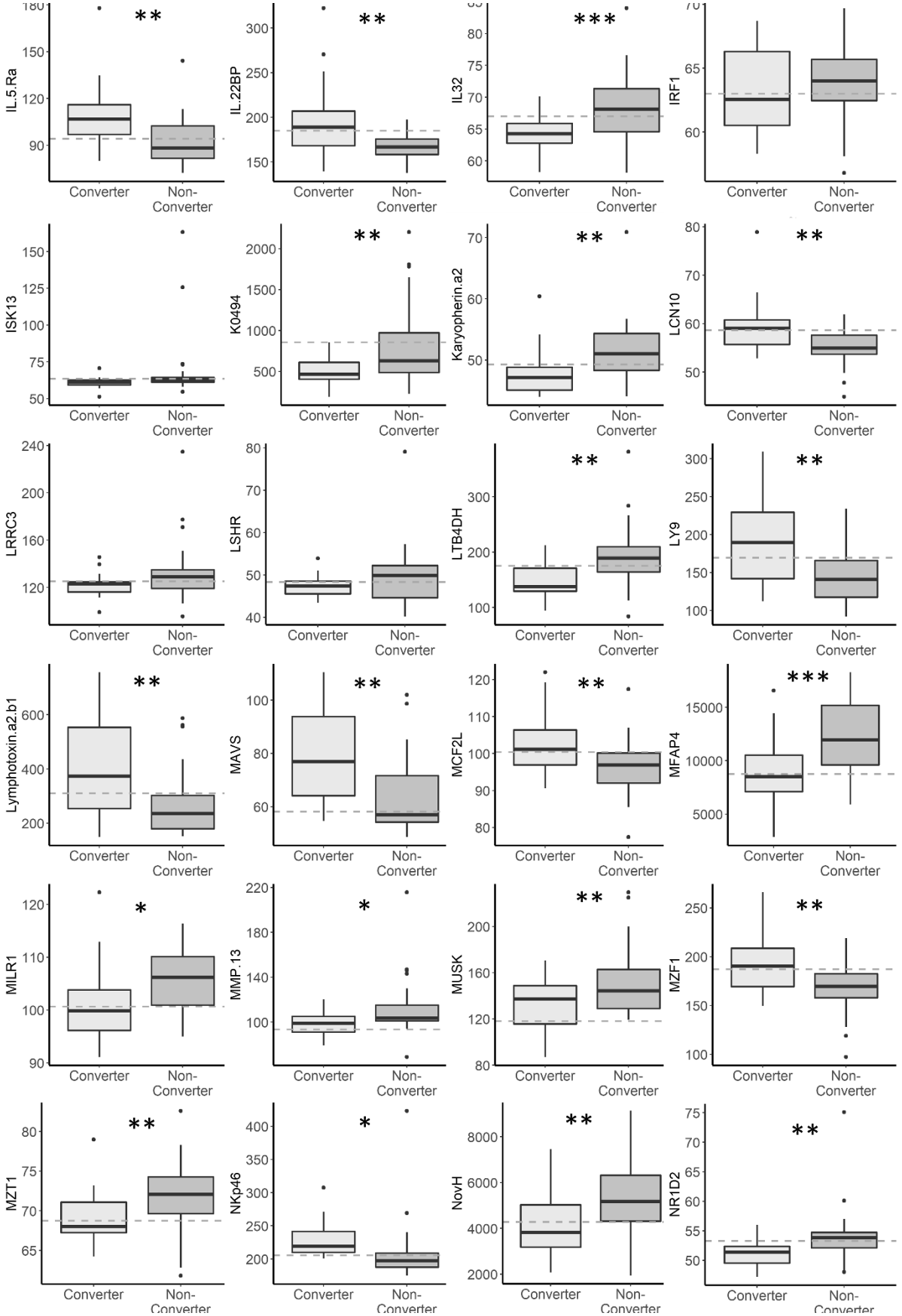
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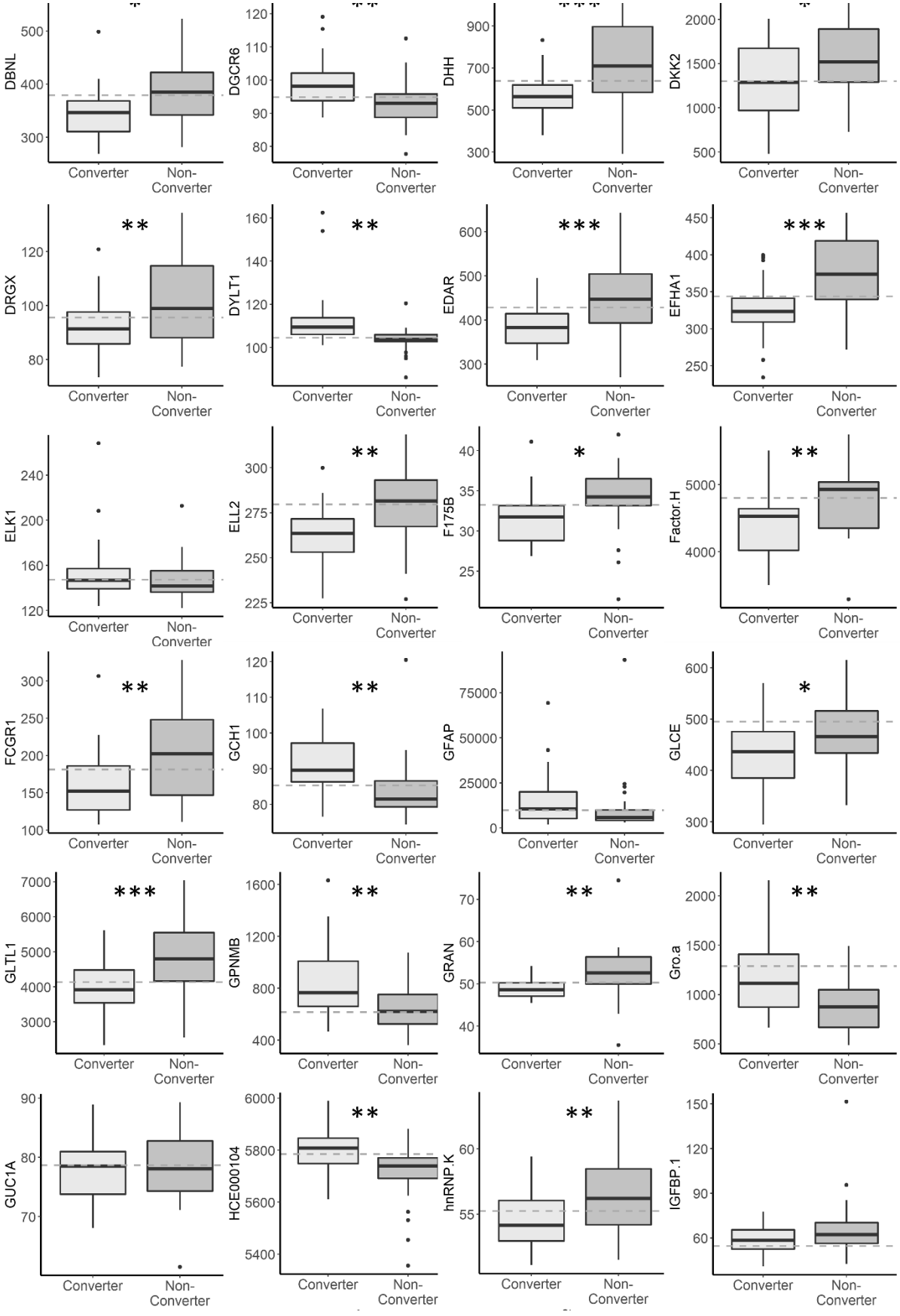
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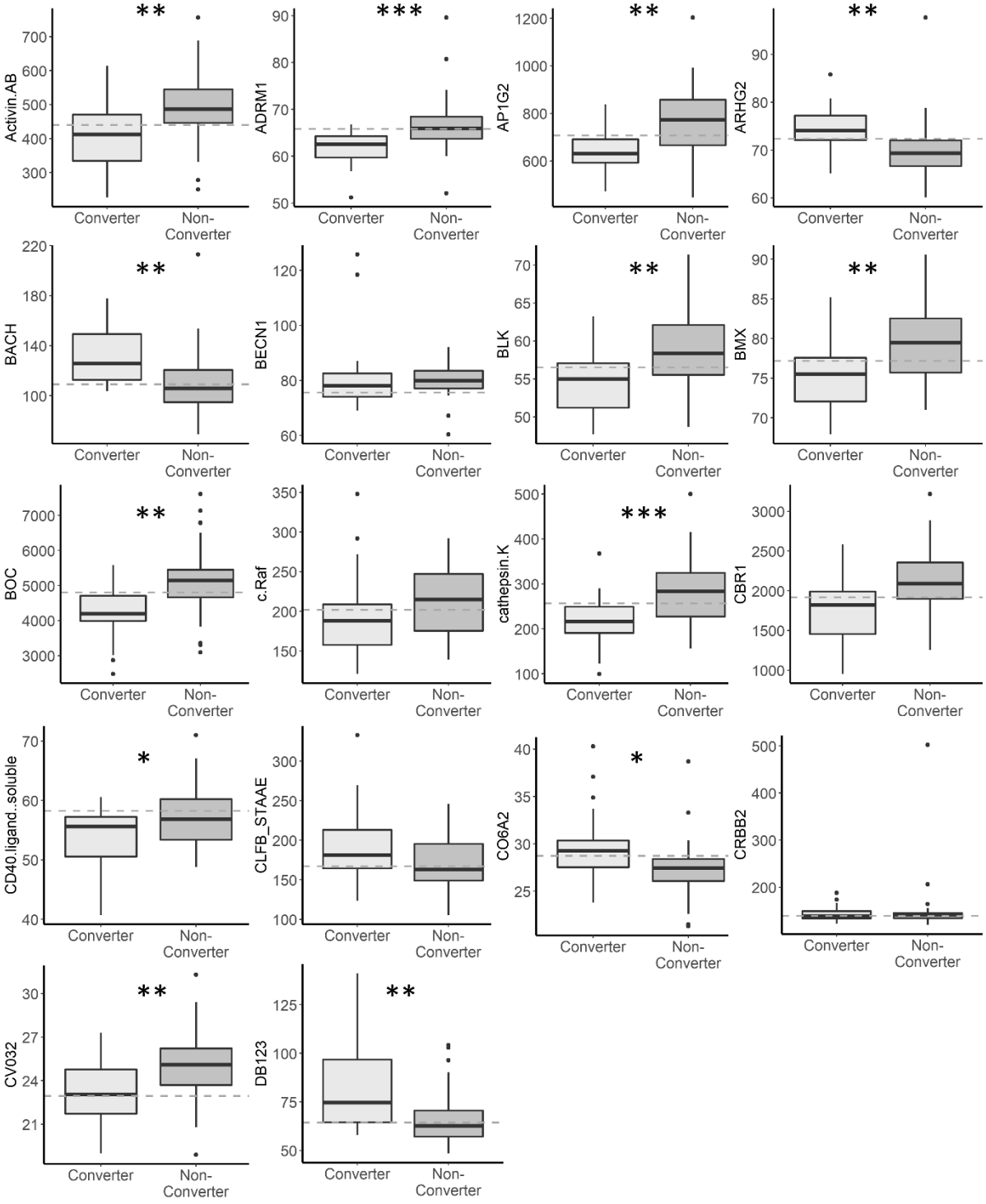

Supplementary Figure 5. Boxplots of the 89 significant discriminatory proteins identified by the OPLS-DA analysis in converters (white) and non-converters (grey). Univariate p-values below 0.05, 0.01, and 0.001 are represented by *, **, *** respectively.

Supplementary Table 1. Predictive performance of 89 identified CSF protein biomarkers identified by multivariate analysis. Biomarkers are listed from highest to lowest AUC. Abbreviations: AUC, area under the curve; CI, confidence interval, Acc, Accuracy; Sens, Sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operator curve.

Uniprot #	Protein	Gene ID	Perturbation	AUC [95% CI]	Acc	Sens	Spec	PPV	NPV	ROC threshold	Odds Ratio	P-value
P63172	Dynein light chain Tctex-type 1	DYNLT1	upregulated in converter	0.83 [0.71-0.95]	78%	92%	67%	69%	91%	104.5	22.00	<0.001
P18887	DNA repair protein XRCC1	XRCC1	upregulated in non-converter	0.84 [0.72-0.95]	80%	82%	76%	84%	73%	69.6	14.40	<0.001
O76036	Natural cytotoxicity triggering receptor 1	NCR1	upregulated in converter	0.79 [0.66-0.92]	80%	92%	69%	72%	91%	205.5	25.56	<0.001
P01210	Proenkephalin-A	PENK	upregulated in non-converter	0.78 [0.65-0.91]	78%	83%	71%	78%	77%	570.2	12.14	0.001
P28676	Grancalcin	GCA	upregulated in non-converter	0.77 [0.63-0.9]	74%	82%	65%	72%	77%	50.4	8.69	0.001
Q6ZSG1	E3 ubiquitin-protein ligase RNF165	RNF165	upregulated in converter	0.84 [0.72-0.95]	72%	81%	63%	69%	77%	219.7	7.48	0.002
O75582	Ribosomal protein S6 kinase alpha-5	RPS6KA5	upregulated in non-converter	0.79 [0.67-0.92]	78%	78%	78%	88%	64%	24.9	12.25	0.002
P06753	Tropomyosin alpha-3 chain	TPM3	upregulated in non-converter	0.69 [0.54-0.84]	78%	100%	65%	63%	100%	150.3	73.33	0.001

Q16186	Proteasomal ubiquitin receptor ADRM1	ADRM1	upregulated in non-converter	0.74 [0.6-0.88]	72%	90%	61%	59%	91%	65.9	14.62	0.003
Q7Z434	Mitochondrial antiviral-signalling protein	MAVS	upregulated in converter	0.73 [0.59-0.87]	70%	90%	59%	56%	91%	58.2	12.86	0.002
P30793	GTP cyclohydrolase 1	GCH1	upregulated in converter	0.74 [0.6-0.88]	76%	85%	67%	72%	82%	85.3	11.50	0.003
Q92974	Rho guanine nucleotide exchange factor 2	ARHGEF2	upregulated in converter	0.74 [0.6-0.88]	76%	81%	70%	78%	73%	72.4	9.52	0.002
P52292	Importin subunit alpha-1	KPNA2	upregulated in non-converter	0.78 [0.65-0.91]	72%	81%	63%	69%	77%	49.3	7.48	0.003
P55083	Microfibril-associated glycoprotein 4	MFAP4	upregulated in non-converter	0.78 [0.65-0.91]	78%	78%	78%	88%	64%	8757.5	12.25	0.003
O00154	Cytosolic acyl coenzyme A thioester hydrolase	ACOT7	upregulated in converter	0.78 [0.65-0.91]	74%	91%	63%	63%	91%	109.2	16.67	0.002
Q14995	Nuclear receptor subfamily 1 group D member 2	NR1D2	upregulated in non-converter	0.68 [0.54-0.83]	72%	90%	61%	59%	91%	53.3	14.62	0.004
Q9HB21	Pleckstrin homology domain-containing family A member 1	PLEKHA1	upregulated in non-converter	0.71 [0.57-0.86]	78%	81%	73%	81%	73%	39.2	11.56	0.01
P19320	Vascular cell adhesion protein 1	VCAM1	upregulated in converter	0.72 [0.58-0.86]	72%	79%	64%	72%	73%	181.1	6.81	0.001
Q9UNE0	Tumor necrosis factor receptor superfamily member EDAR	EDAR	upregulated in non-converter	0.72 [0.58-0.86]	72%	84%	62%	66%	82%	428.6	8.59	0.003

P43235	Cathepsin K	CTSK	upregulated in non-converter	0.74 [0.6-0.88]	72%	84%	62%	66%	82%	257.1	8.59	0.01
Q8IYU8	Calcium uptake protein 2, mitochondrial	MICU2	upregulated in non-converter	0.7 [0.55-0.85]	74%	82%	65%	72%	77%	344.1	8.69	0.005
P24001	Interleukin-32	IL32	upregulated in non-converter	0.72 [0.57-0.86]	72%	90%	61%	59%	91%	67	14.62	0.004
Q14914	Prostaglandin reductase 1	PTGR1	upregulated in non-converter	0.75 [0.61-0.89]	74%	85%	64%	69%	82%	175.5	9.90	0.002
O75843	AP-1 complex subunit gamma-like 2	AP1G2	upregulated in non-converter	0.75 [0.62-0.89]	74%	85%	64%	69%	82%	708.5	9.90	0.01
Q01344	Interleukin-5 receptor subunit alpha	IL5RA	upregulated in converter	0.75 [0.61-0.89]	74%	82%	65%	72%	77%	94.3	8.69	0.01
Q8N688	Beta-defensin 123	DEFB123	upregulated in converter	0.79 [0.67-0.92]	69%	80%	59%	63%	77%	64.4	5.67	0.01
Q8N428	Polypeptide N-acetylgalactosaminyltransferase 16	GALNT16	upregulated in non-converter	0.75 [0.62-0.89]	74%	78%	68%	78%	68%	4138.8	7.65	0.005
P01374	Lymphotoxin-alpha	LTA	upregulated in converter	0.76 [0.62-0.89]	74%	78%	68%	78%	68%	310.4	7.65	0.005
Q9BWV1	Brother of CDO	BOC	upregulated in non-converter	0.72 [0.57-0.86]	74%	85%	64%	69%	82%	4808.5	9.90	0.01
P35442	Thrombospondin-2	THBS2	upregulated in non-converter	0.71 [0.56-0.85]	72%	84%	62%	66%	82%	39830.9	8.59	0.004

Q9HC23	Prokineticin-2	PROK2	upregulated in non-converter	0.73 [0.59-0.87]	76%	81%	70%	78%	73%	137.4	9.52	0.005
Q08AG7	Mitotic-spindle organizing protein 1	MZT1	upregulated in non-converter	0.74 [0.6-0.88]	76%	79%	71%	81%	68%	68.8	9.29	0.01
P51813	Cytoplasmic tyrosine-protein kinase BMX	BMX	upregulated in non-converter	0.74 [0.6-0.88]	69%	78%	59%	66%	73%	77.2	5.09	0.02
Q969J5	Interleukin-22 receptor subunit alpha-2	IL22RA2	upregulated in converter	0.75 [0.61-0.88]	76%	76%	76%	88%	59%	185.1	10.11	0.01
Q6JVE6	Epididymal-specific lipocalin-10	LCN10	upregulated in converter	0.77 [0.64-0.91]	74%	74%	75%	88%	55%	58.7	8.40	0.004
Q96PF2	Testis-specific serine	TSSK2	upregulated in non-converter	0.72 [0.58-0.86]	72%	74%	68%	81%	59%	79.8	6.26	0.005
P08603	Complement factor H	CFH	upregulated in non-converter	0.78 [0.65-0.91]	72%	90%	61%	59%	91%	4803.9	14.62	0.01
O00472	RNA polymerase II elongation factor ELL2	ELL2	upregulated in non-converter	0.79 [0.66-0.92]	69%	89%	57%	53%	91%	279.8	11.33	0.01
O00300	Tumor necrosis factor receptor superfamily member 11B	TNFRSF11B	upregulated in non-converter	0.71 [0.57-0.86]	74%	85%	64%	69%	82%	10188.4	9.90	0.01
O43323	Desert hedgehog protein	DHH	upregulated in non-converter	0.72 [0.57-0.86]	72%	84%	62%	66%	82%	639	8.59	0.01
Q14956	Transmembrane glycoprotein NMB	GPNMB	upregulated in converter	0.76 [0.62-0.9]	65%	84%	54%	50%	86%	616.9	6.33	0.02

Q15018	BRISC complex subunit Abraxas 2	ABRAXAS2	upregulated in non-converter	0.72 [0.57-0.86]	76%	83%	68%	75%	77%	33.3	10.20	0.01
P51451	Tyrosine-protein kinase Blk	BLK	upregulated in non-converter	0.72 [0.58-0.87]	69%	78%	59%	66%	73%	56.6	5.09	0.02
Q7Z6M3	Allergin-1	MILR1	upregulated in non-converter	0.73 [0.59-0.87]	72%	76%	67%	78%	64%	100.7	6.25	0.01
P16152	Carbonyl reductase [NADPH] 1	CBR1	upregulated in non-converter	0.77 [0.63-0.9]	70%	75%	64%	75%	64%	1917.3	5.25	0.01
Q9HBG7	T-lymphocyte surface antigen Ly-9	LY9	upregulated in converter	0.76 [0.63-0.9]	70%	75%	64%	75%	64%	169.5	5.25	0.01
Q14129	Protein DGCR6	DGCR6	upregulated in converter	0.74 [0.6-0.88]	72%	79%	64%	72%	73%	94.9	6.81	0.01
P08476/P09529	Inhibin beta A/B chain	INHBA/INHBB	upregulated in non-converter	0.75 [0.62-0.89]	74%	78%	68%	78%	68%	440.1	7.65	0.03
P48745	CCN family member 3	CCN3	upregulated in non-converter	0.73 [0.59-0.87]	72%	77%	65%	75%	68%	4284.3	6.43	0.02
Q96IQ7	V-set and immunoglobulin domain-containing protein 2	VSIG2	upregulated in non-converter	0.73 [0.58-0.87]	70%	77%	63%	72%	68%	246.5	5.48	0.01
Q13356	RING-type E3 ubiquitin-protein ligase PPIL2	PPIL2	upregulated in converter	0.66 [0.51-0.81]	72%	74%	68%	81%	59%	94.1	6.26	0.03
Q99969	Retinoic acid receptor responder protein 2	RARRES2	upregulated in non-converter	0.73 [0.59-0.87]	74%	73%	79%	91%	50%	4102.7	9.67	0.01

Q9H4I9	Essential MCU regulator, mitochondrial	SMDT1	upregulated in non-converter	0.78 [0.65-0.91]	74%	73%	79%	91%	50%	23	9.67	0.02
Q16585	Beta-sarcoglycan	SGCB	upregulated in converter	0.77 [0.64-0.9]	72%	72%	73%	88%	50%	110.2	7.00	0.01
P09341	Growth-regulated alpha protein	CXCL1	upregulated in converter	0.73 [0.58-0.87]	72%	70%	82%	94%	41%	1289.6	10.38	0.03
P12314	High affinity immunoglobulin gamma Fc receptor I	FCGR1A	upregulated in non-converter	0.78 [0.65-0.91]	70%	79%	62%	69%	73%	181.2	5.87	0.01
P12110	Collagen alpha-2(VI) chain	COL6A2	upregulated in converter	0.67 [0.52-0.82]	72%	74%	68%	81%	59%	28.8	6.26	0.01
Q76M96	Coiled-coil domain-containing protein 80	CCDC80	upregulated in non-converter	0.71 [0.56-0.85]	72%	73%	71%	84%	55%	293.1	6.48	0.03
O15068	Guanine nucleotide exchange factor DBS	MCF2L	upregulated in converter	0.71 [0.56-0.85]	69%	71%	63%	78%	55%	100.4	4.29	0.02
P45452	Collagenase 3	MMP13	upregulated in non-converter	0.72 [0.57-0.86]	74%	70%	90%	97%	41%	93.5	21.46	0.02
Q1W4C9	Serine protease inhibitor Kazal-type 13	SPINK13	upregulated in non-converter	0.62 [0.46-0.77]	63%	88%	53%	44%	91%	63.6	7.78	0.02
P28698	Myeloid zinc finger 1	MZF1	upregulated in converter	0.74 [0.59-0.88]	74%	75%	72%	84%	59%	187.3	7.80	0.03
O75071	EF-hand calcium-binding domain-containing protein 14	EFCAB14	upregulated in non-converter	0.75 [0.62-0.89]	61%	100%	51%	34%	100%	857.1	23.05	0.03

Q9BWV2	Spermatogenesis-associated protein 9	SPATA9	upregulated in non-converter	0.73 [0.59-0.87]	63%	83%	53%	47%	86%	235.6	5.59	0.02
Q92583	C-C motif chemokine 17	CCL17	upregulated in converter	0.68 [0.53-0.83]	76%	73%	85%	94%	50%	106	15.00	0.03
O94923	D-glucuronyl C5-epimerase	GLCE	upregulated in non-converter	0.56 [0.41-0.72]	61%	92%	51%	38%	95%	495.1	12.60	0.04
Q9UJU6	Drebrin-like protein	DBNL	upregulated in non-converter	0.66 [0.51-0.81]	67%	82%	56%	56%	82%	379.5	5.79	0.03
A6NNA5	Dorsal root ganglia homeobox protein	DRGX	upregulated in non-converter	0.59 [0.44-0.75]	69%	78%	59%	66%	73%	95.7	5.09	0.01
P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	upregulated in non-converter	0.57 [0.41-0.73]	67%	73%	58%	69%	64%	55.3	3.85	0.06
O15146	Muscle, skeletal receptor tyrosine-protein kinase	MUSK	upregulated in non-converter	0.73 [0.59-0.87]	74%	70%	100%	100%	36%	118.1	36.57	0.02
P16035	Metalloproteinase inhibitor 2	TIMP2	upregulated in non-converter	0.63 [0.47-0.78]	67%	85%	56%	53%	86%	1771.4	7.18	0.02
Q9UN72	Protocadherin alpha-7	PCDHA7	upregulated in non-converter	0.68 [0.53-0.83]	70%	77%	63%	72%	68%	450.2	5.48	0.04
Q9BS26	Endoplasmic reticulum resident protein 44	ERP44	upregulated in non-converter	0.67 [0.52-0.82]	65%	78%	55%	56%	77%	548.4	4.37	0.04
Q9UBU2	Dickkopf-related protein 2	DKK2	upregulated in non-converter	0.64 [0.48-0.79]	67%	71%	60%	75%	55%	1302.6	3.60	0.04

P35443	Thrombospondin-4	THBS4	upregulated in non-converter	0.68 [0.53-0.83]	70%	69%	75%	91%	41%	229.3	6.69	0.03
P29965	CD40 ligand	CD40LG	upregulated in non-converter	0.65 [0.5-0.8]	61%	87%	51%	41%	91%	58.3	6.84	0.06
O86476	OS=Staphylococcus aureus (strain Newman) OX=426430 GN=clfB PE=1 SV=2	None	upregulated in converter	0.66 [0.5-0.81]	65%	76%	55%	59%	73%	167.3	3.90	0.07
P04049	RAF proto-oncogene serine	RAF1	upregulated in non-converter	0.65 [0.5-0.8]	65%	74%	56%	63%	68%	202.2	3.57	0.08
Q9BY71	Leucine-rich repeat-containing protein 3	LRRC3	upregulated in non-converter	0.73 [0.58-0.87]	67%	82%	56%	56%	82%	125.4	5.79	0.08
P22888	Lutropin-choriogonadotropic hormone receptor	LHCGR	upregulated in non-converter	0.69 [0.55-0.84]	67%	77%	57%	63%	73%	48.4	4.44	0.13
P14136	Glial fibrillary acidic protein	GFAP	upregulated in converter	0.7 [0.56-0.85]	69%	73%	62%	75%	59%	9851.5	4.33	0.13
P08833	Insulin-like growth factor-binding protein 1	IGFBP1	upregulated in non-converter	0.55 [0.39-0.71]	67%	68%	63%	81%	45%	54.7	3.61	0.1
Q14457	Beclin-1	BECN1	upregulated in converter	0.65 [0.5-0.81]	33%	36%	33%	16%	59%	75.7	0.27	0.21
Q01968	Inositol polyphosphate 5-phosphatase OCRL-1	OCRL	upregulated in non-converter	0.68 [0.53-0.83]	67%	69%	61%	78%	50%	218.3	3.57	0.3
P10914	Interferon regulatory factor 1	IRF1	upregulated in non-converter	0.69 [0.54-0.84]	67%	72%	59%	72%	59%	63	3.69	0.33

P19419	ETS domain-containing protein Elk-1	ELK1	upregulated in converter	0.48 [0.32-0.64]	61%	67%	52%	69%	50%	147.2	2.20	0.36
P51814	Zinc finger protein 41	ZNF41	upregulated in non-converter	0.52 [0.36-0.68]	56%	68%	47%	47%	68%	83.5	1.89	0.53
P43320	Beta-crystallin B2	CRYBB2	upregulated in non-converter	0.46 [0.31-0.62]	59%	67%	50%	63%	55%	139.4	2.00	0.76
P43080	Guanylyl cyclase-activating protein 1	GUCA1A	upregulated in non-converter	0.59 [0.43-0.74]	46%	56%	38%	44%	50%	78.7	0.78	0.88

Supplementary Table 2. Predictive performance of the top 20 identified CSF protein biomarkers with highest sensitivity compared to the performance of OCGB status. Proteins are listed from highest to lowest sensitivity. Abbreviations: AUC, area under the curve; CI, confidence interval, Acc, Accuracy; Sens, Sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operator curve.

	AUC [95% CI]	Acc	Sens	Spec	PPV	NPV	ROC threshold	Odds Ratio	P-value
TPM3	0.69 [0.54-0.84]	78%	100%	63%	65%	100%	150.3	73.33	0.001
EFCAB14	0.75 [0.62-0.89]	61%	100%	34%	51%	100%	857.1	23.05	0.03
OCGB status	0.66 [0.5-0.81]	59%	100%	31%	50%	100%	0.5	21.00	0.01
GLCE	0.56 [0.41-0.72]	0.61	0.95	0.38	0.51	0.92	495.1	12.60	0.04
DYNLT1	0.83 [0.71-0.95]	78%	91%	69%	67%	92%	104.5	22.00	<0.001
NCR1	0.79 [0.66-0.92]	80%	91%	72%	69%	92%	205.5	25.56	<0.001
ADRM1	0.74 [0.6-0.88]	72%	91%	59%	61%	90%	65.9	14.62	0.003
MAVS	0.73 [0.59-0.87]	70%	91%	56%	59%	90%	58.2	12.86	0.002
ACOT7	0.78 [0.65-0.91]	74%	91%	63%	63%	91%	109.2	16.67	0.002
NR1D2	0.68	72%	91%	59%	61%	90%	53.3	14.62	0.004

	[0.54-0.83]								
IL32	0.72 [0.57-0.86]	72%	91%	59%	61%	90%	67	14.62	0.004
CFH	0.78 [0.65-0.91]	72%	91%	59%	61%	90%	4803.9	14.62	0.01
ELL2	0.79 [0.66-0.92]	69%	91%	53%	57%	89%	279.8	11.33	0.01
SPINK13	0.62 [0.46-0.77]	63%	91%	44%	53%	88%	63.6	7.78	0.02
CD40LG	0.65 [0.5-0.8]	61%	91%	41%	51%	87%	58.3	6.84	0.06
GPNMB	0.76 [0.62-0.9]	65%	86%	50%	54%	84%	616.9	6.33	0.02
SPATA9	0.73 [0.59-0.87]	63%	86%	47%	53%	83%	235.6	5.59	0.02
TIMP2	0.63 [0.47-0.78]	67%	86%	53%	56%	85%	1771.4	7.18	0.02
GCH1	0.74 [0.6-0.88]	76%	82%	72%	67%	85%	85.3	11.50	0.003
EDAR	0.72 [0.58-0.86]	72%	82%	66%	62%	84%	428.6	8.59	0.003
CTSK	0.74 [0.6-0.88]	72%	82%	66%	62%	84%	257.1	8.59	0.01
PTGR1	0.75 [0.61-0.89]	74%	82%	69%	64%	85%	175.5	9.90	0.002

Detailed statistical methods.

Stage 1. Model validation

OPLS-DA models were optimized by internal 7-fold cross-validation. The quality of classification was assessed using a 10-fold external cross-validation scheme with 1000 repetitions in total, correcting for unequal class sizes. This validation scheme involves multiple iterations of splitting the data into training and testing sets, which ensures that any discrimination observed in the models cannot have occurred by chance. The training data is used to estimate the model parameters (number of components, R^2 – the 'goodness' of fit, and Q^2 – the internal predictive performance of the model on the training dataset) and learn the underlying discriminatory patterns between the groups under consideration, whereas the independent test set is employed to assess the accuracy, sensitivity, specificity, and generalizability of the trained models in the ensemble. We quantified the outcome of the cross validation by calculating the accuracy, sensitivity, and specificity of each model (n = 1000) from the predicted classifications of the external independent test set, which was not used to build each model. It is important to appreciate that the classifier (OPLS-DA) was blinded to each test set when training each model. This validation scheme tends to avoid over-fitting and helps assess the generalizability of the model to previously unseen datasets. For an exhaustive discussion on validation of this approach see Arlot and Celisse (2010). These values were compared with those of a null distribution (obtained from randomly permuting the classifications) using the two-sided Kolmogorov-Smirnov test (significant if p-value 0.05 or less).

Stage 2. Prediction and identification of discriminatory metabolites.

If stage 1 of the analysis reveals that the OPLS-DA classifier performs significantly better than chance, the separation observed between classes is confirmed and it is valid to interrogate metabolites driving the separation and to use the data for prediction of additional samples. Discriminatory variables were identified by calculating the average of the variable importance (VIP) scores of the ensemble of models. A VIP cut-off of 1.5 was used to identify the most important variables driving the separation between classes. Two-sample t-tests and ROC analysis was applied to each of the variables identified as discriminatory by the multivariate analysis to investigate the diagnostic accuracy of each biomarker in isolation. The p-values obtained were then corrected for multiple comparisons using the Bonferroni correction.