*Supplementary sheet
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Supplementary sheet

Protein identification

2D PAGE comparison

The protein concentrations of the CSF samples were obtained using the Bradford assay. CSF volumes equivalent to 75 µg (for silver staining) or 400 µg (Coomassie brilliant blue, CBB, staining) of protein sample was required for 2D PAGE. For each sample, 2-D PAGE was repeated in duplicate.

MALDI-TOF

Following the selection of the spots of interest, they were manually excised from the stained gels and subjected to in-gel tryptic digestion. Silver stained samples were destained using a 1:1 mix of 100mM sodium thiosulphate and 30 mM potassium ferracyanide. Protein spots of interest were manually excised from the gel using pen picking. Spots were equilibrated in 200mM ammonium bicarbonate (ambic) and dehydrated using a 2:1 acetonitrile (ACN):ambic (25mM) solution. Spots were vacuum centrifuged to dryness and a 12.5 ng/µl solution of sequencing grade, modified trypsin (Promega, Madison, WI, USA) in ambic (25mM) was applied to the gel spot. In-gel digestion was carried out overnight at 37°C. Peptide extraction was carried out by sonication (10 min) followed by centrifugation (10000 RPM) and transfer of supernatant to a new microcentrifuge tube. Neat ACN was added to the gel spot and incubated for a further 30 minutes at 37°C. Peptide extraction was repeated and the supernatant pooled with the earlier supernatant. The final pooled supernatant was vacuum centrifuged to 3 µl. 0.5 µl of the sample to be analyzed was spotted onto the MALDI target plate. The remainder was concentrated using a Zip Tip C18 (Millipore) and also spotted onto the

target plate. 0.5 µl of α-cyano-4-hydroxycinnamic acid (Sigma) solution was layered over the sample spot. Sample spots were allowed to air dry before insertion into the MALDI-TOF. The MALDI-TOF spectra of the peptides were obtained with an Axima CFR plus mass spectrometer (Kratos analytical, Manchester UK) in 2 GHz reflectron mode. Near spot calibration was performed using a 5 point calibration mix: Bradykinin [1-5] (572.7 Da), Angiotensin II (1046.2 Da), Neurotensin (1672.9 Da), ACTH [18-39] (2465.7 Da) and Bovine Insulin chain B (3495.9 Da) (Laserbiolabs, France). Acquisition and data processing were controlled by Launchpad software V. 2.4 (Kratos Analytical, Shimadzu, Manchester, UK). The positive control used was BSA solution and was subject to the same digestion procedure. The negative control used was a blank gel piece sourced from the same gel under analysis. Peaks from the negative control were deducted from the raw peak list to create the final peak list. The PMF obtained for each protein digest was analyzed using the Swiss Prot and MSDB non-redundant protein databases against *Homo sapiens* and *Streptococcus pneumoniae* with MASCOT software (Matrix Science, London, UK, at http://www.matrixscience.com).

LC-coupled ESI MS/MS

Digestion was carried out as described for MALDI-TOF. Samples were mixed with 5% formic acid in 50% ACN. The separation and analysis of digested peptides were performed using reverse phase (RP) capillary high performance liquid chromatography (HPLC, Dionex ultimate 3000) directly coupled to a Finnigan LCQ IT mass spectrometer (Thermofisher). A C18 PepMap100 solid-phase extraction m-Precolumn cartridge (particle size 5 mm, pore size 100 Å, 300 mm inner diameter) (Dionex) trapping column and Nano-column PepMap C18 reversed-phase material (particle size 3 mm, pore size

100Å, 75 mm inner diameter, Dionex) resolving column were placed in-line. Peptides were bound and preconcentrated in the trapping column using 0.1% formic acid in 2.5% ACN. The eluting gradient was 2–90% v/v ACN in 0.1% v/v formic acid for 50 min at a flow rate of 0.3 µL/min. Eluent from the capillary column was directly sprayed into the ion trap mass spectrometer. All the data were collected in centroid mode using "triple play" settings (i.e. a full mass scan at mass range of 400– 1500 Da (*m/z*), determination of the charge states of an ion on zoom scan, and then acquisition of the MS/MS spectrum of each ion on a full MS/MS scan, with collision energy preset at a value of 55%). Calibration of the mass spectrometer was carried before each batch of samples using human glufibrinopeptide B (Sigma Aldrich).

Mascot search

Criteria for the positive identification of the proteins with MALDI were: (1) Scores above the MOWSE statistical threshold value in MASCOT using the Swiss Prot and MSDB database. (2) Protein must be identified in at least two of the three replicates by a minimum of five peptides in MALDI-TOF. (3) Protein sequence must have a minimum of 10% sequence coverage.

LC-MS/MS *raw* file spectra were exported as *dta* (text format) files using LCQ_DTA *raw* file converter software (Matrix science) using standard settings. *Dta* files were merged into a single MASCOT generic format (*mgf*) file. Criteria for positive identification of the proteins with LC-MS/MS: (1) Scores above 100 according to the MOWSE statistical threshold value in MASCOT using the Swiss Prot database were considered significant. (2) Protein must be identified in at least two of the three replicates, by a minimum of two peptides (unless previous identification with MALDI

TOF). The threshold ions scores suggested by MASCOT for confident PMF identification in Swiss Prot and MSDB databases were 55 and 63 ($p < 0.05$), respectively. The threshold score for MS/MS data was 31 for individual peptides.

Table 2. Functional clustering of identified proteins

Functional category	*Protein*	*Up or Down regulation*	*Fold Expression in Non-survivors*	*Expression Median*		*Confidence interval*	
				Survivors	**Non-survivors**	**Survivors**	**Non-survivors**
Cellular defense proteins	Complement C3 precursor	↓	4.77	0.051	0.010	0.049	0.006
	Chitotriosidase	↑	5.14	0.016	0.124	0.029	0.07
	Complement C1q tumor necrosis factor-related protein 9	↑	3.42	0.026	0.074	0.012	0.044
Chaperones	T-complex protein 1 subunit zeta	↓	2.75	0.015	0.005	0.014	0.005
Metabolic enzymes	Phosphoglucomutase-like protein 5	↓	4.87	0.031	0.010	0.032	0.006
	Glyoxalase domain-containing protein 4	↓	3.57	0.025	0.013	0.052	0.005
	26S protease regulatory subunit 7	↑	2.46	0.008	0.013	0.004	0.022
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	↑	2.76	0.006	0.004	0.005	0.019
Translation	Retinoic acid receptor RXR-gamma	↓	2.46	0.067	0.028	0.029	0.008
	Cleavage stimulation factor (CSTFT) 64 kDa subunit, tau variant	↑	3.30	0.023	0.092	0.026	0.067
	Heterogeneous nuclear ribonucleoproteins C1/C2	↑	2.46	0.025	0.055	0.013	0.032
	Zinc finger protein 179	↑	2.02	0.036	0.049	0.014	0.026
	Zinc finger protein 1	↓	2.02	0.188	0.053	0.072	0.039
	Eukaryotic translation initiation factor 2, subunit 2 beta	↓	2.18	0.050	0.026	0.022	0.015

Table 1 continued.

Functional category	*Protein*	*Up or Down regulation*	*Fold Expression in Non-survivors*	*Expression Median*		*Confidence interval*	
				Survivors	**Non-survivors**	**Survivors**	**Non-survivors**
Transporters	Serotransferrin	↓	5.47	0.100	0.017	0.051	0.019
	Solute carrier family 25 (member 16)	↑	3.97	0.005	0.006	0.005	0.024
Glycoproteins	Beta-2-glycoprotein 1 precursor	↑	3.61	0.011	0.032	0.023	0.063
	Zinc alpha 2-glycoprotein precursor	↓	2.87	0.033	0.021	0.043	0.012
G proteins of the Ras family	Ras-related protein Rab-37	↓	3.82	0.005	0.002	0.011	0.004
Globins	Haptoglobin	↑	2.91	0.012	0.026	0.023	0.041
Kinases	Brain-enriched guanylate kinase-associated protein (BEGAIN)	↓	5.01	0.035	0.016	0.102	0.011
	Pyruvate kinase	↑	2.06	0.025	0.048	0.017	0.055
Proteases	Tryptophan/serine protease	↑	3.07	0.003	0.012	0.006	0.013
Phosphatases	Lysosomal Acid Phosphatase	↑	2.68	0.006	0.019	0.018	0.028
	Serine/Threonine phosphatase 2-alpha 65K regulatory chain	↓	2.68	0.118	0.032	0.029	0.035
	Serine/threonine/tyrosine-interacting-like protein 1	↓	2.24	0.217	0.089	0.063	0.025
Membrane and skeletal proteins	Fibrinogen	↓	2.51	0.032	0.010	0.021	0.007
	Fascin	↑	3.02	0.050	0.053	0.014	0.056
	Mutant desmin	↑	2.71	0.024	0.049	0.006	0.026
	Ankyrin repeat domain-containing protein 42	↓	2.51	0.056	0.028	0.027	0.019

Table 1 contd.

Functional category	*Protein*	*Up or Down regulation*	*Fold Expression in Non-survivors*	*Expression Median*		*Confidence interval*	
				Survivors	**Non-survivors**	**Survivors**	**Non-survivors**
Others	Alpha 1 antitrypsin precursor	↓	2.97	0.014	0.006	0.013	0.011
	Nuclear localized factor 1	↓	2.09	0.062	0.022	0.096	0.048
	Human Serum Albumin	↓	2.01	0.236	0.089	0.223	0.149
Unknown	Cancer associated gene 1	↓	2.45	0.058	0.007	0.036	0.036

The table above shows the functional clustering of the proteins identified from mass spectrometry. The expression of each protein was based on the normalized volume of each of the protein spots when identified as a match in all the gels. This was then used to create an averaged gel which involved comparing the non-survivors to the survivor gels. The median of each normalized volume of the proteins from the samples is shown along with the 95% confidence intervals for the normalized volumes.