Plasma proteome profiling of healthy subjects undergoing bed rest reveals unload-dependent changes linked to muscle atrophy.

Marta Murgia*, Lorenza Brocca, Elena Monti, Martino V. Franchi, Maximilian Zwiebel, Sophia Steigerwald, Emiliana Giacomello, Roberta Sartori, Sandra Zampieri, Giovanni Capovilla, Mladen Gasparini, Gianni Biolo, Marco Sandri, Matthias Mann and Marco V. Narici

*Correspondence to: Marta Murgia, Department of Biomedical Sciences, University of Padova, Via Ugo Bassi, 58/B, 35131, Padua, Italy. Tel. +39 049 827 6031, e-mail: marta.murgia@unipd.it

Supporting information

Supplementary methods

Inclusion and exclusion criteria of the subject cohorts

Bed rest cohort. Inclusion criteria: age between 18 and 33 who gave informed consent. Moderately physically active (<90 minutes of low-medium intensity exercise/day) but not engaged in competitive sport. Exclusion criteria: regular alcohol consumption; ferromagnetic implants; history of deep vein thrombosis; skeletal, neuromuscular, metabolic and cardiovascular disease conditions, regular assumption of medications

Cancer patient cohort. Inclusion criteria: individuals >18 years who gave informed consent. Exclusion criteria: presence of active inflammatory or infective diseases, known myopathies, or viral infections (such as hepatitis c virus, hepatitis B virus, and HIV). Control patients were excluded if having history of cancer.

Plasma and serum collection

For the bed rest cohort, blood was drawn between 7 and 7.30 am after overnight fasting and collected in S-Monovette tubes containing EDTA (Sarsted). Plasma was prepared by centrifugation (2000×g for 10 minutes at 4 °C) in the Central Laboratory of the Izola General Hospital and subsequently kept refrigerated for less than 30 minutes, before being aliquoted and snap-frozen in dry ice. For the cancer patient cohort, serum samples were prepared from blood stored in collection tubes left at room temperature for 30 min and subsequently centrifuged at 3000×g for 10 min at 4 °C. Supernatant, corresponding to the serum fraction, was aliquoted and stored at −80 °C.

Measurements of plasma cholesterol, insulin and glucose concentration were carried out by a certified clinical laboratory connected to the Polyclinic of Trieste (Italy). The estimate of insulin resistance by HOMA-IR was calculated with the formula fasting serum insulin (μU/ml) fasting plasma glucose (mmol/l)/22.5, as described [1].

Western blot

Plasma samples were run on a 4–12% Bis-Tris polyacrylamide gels using MOPS as a trailing ion in the buffer (Invitrogen). For the depletion of twelve top abundant proteins from plasma we used a spin column-based kit (A36369, Thermo Scientific). Proteins were transferred to a nitrocellulose membrane (Whatman) using Towbin buffer with 20% (v/v) methanol in a semi-dry transfer apparatus (Hoefer) and reversibly stained with Ponceau S for loading control. Membranes were incubated with the primary antibody in TBS buffer containing 0.1% Tween-20 and 5% (w/v) skim milk powder. A rabbit monoclonal anti-Lumican antibody was used (EPR22511-63, Abcam).

Measurement of fiber cross-sectional area

Air dried sections were blocked for 1 hour at room temperature with 5% normal horse serum in PBS to avoid non-specific binding of the antibodies, and incubated with primary antibodies for myosin heavy chain isoforms in a humidified chamber at room temperature for 2 hours. Specimens were washed three times with PBS and then incubated 1 hour with an anti-mouse biotinylated secondary antibody raised in horse and washed and incubated with VECTASTAIN ABC Reagent (all from Vector Laboratories, Burlingame, CA USA) for 30 minutes. Samples were incubated in Diamino Benzidine solution (SigmaFastDAB; Sigma Aldrich) until development of staining. Specimens were rinsed in water, dehydrated with 95% and absolute ethanol alcohol, followed by clarification in BioClear (BioOptica, Milano, Italy), and mounted with a resinous medium. Images were acquired with a Leica DM500 microscope (Leica, Germany) equipped with a Leica ICC50 digital camera (Leica, Germany). Images were processed with Fiji free software, and the cross-sectional area of the fibers was determined by carefully drawing a polygon around each fiber as described [2].

Muscle size measurements with magnetic resonance imaging

MRI data for the measurement of muscle volume were acquired on a 3T scanner (Magnetom Skyra, Siemens, Erlangen, Germany) using the following acquisition parameters: sequence: vibe, TR/TE: 7.8/3.69 ms, flip angle: 20°, field of view: 450 × 337.5 mm, voxel size: 0.9 × 0.9 × 6 mm, no inter-slice gap, slice thickness: 6 mm, readout-bandwidth 320 Hz/pixel (278 kHz). Images were analyzed using the OsiriX DICOM image analysis software (Version 11; Pixmeo Sarl). For further details see Monti et al, J. Physiol. 2021;599:3037-61.

Protein networks

Functional network analysis was performed using the interaction database STRING, version 11.5 [3]. We selected medium to high confidence (0.5–0.7) and filtered interactions keeping only the ones derived from physical interaction, coexpression and data mining. The DAVID database was used to analyze annotation enrichments in networks [4].

Supporting information references

1. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28:412-9.
2. Giacomello E, Crea E, Torelli L, Bergamo A, Reggiani C, Sava G, et al. Age Dependent Modification of the Metabolic Profile of the Tibialis Anterior Muscle Fibers in C57BL/6J Mice. Int J Mol Sci. 2020;2.
3. von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B. STRING: a database of predicted functional associations between proteins. Nucleic Acids Res. 2003;31:258-61.
4. Jiao X, Sherman BT, Huang da W, Stephens R, Baseler MW, Lane HC, et al. DAVID-WS: a stateful web service to facilitate gene/protein list analysis. Bioinformatics. 2012;28:1805-6.

Supplementary tables

Table S1 Biometric data of the ten subjects recruited for the bed rest study

	Bed rest study biometric data					
		BR0		**BR10**		
Subject	**Age (years)**	**Height (cm)**	**Weight (Kg)**	**Height (cm)**	**Weight (Kg)**	**Weight difference BR10-BR0**
S1	33	185.5	87.6	186.5	85.4	-2.2
S2	21	181.5	69.9	180.7	70.4	0.5
S3	19	178	67.5	179	66.8	-0.7
S4	21	182.5	73.4	183.4	69.9	-3.5
S5	18	189.1	96.8	189.8	92.7	-4.1
S6	25	177.8	77.5	178	78.5	1
S7	28	178.5	88.2	179	87.3	-0.9
S8	24	181.7	71	182	69.8	-1.2
S9	21	181	75.5	180.5	72.5	-3
S10	20	176.2	67.7	178.3	66.3	-1.4

BR0, before bed rest; BR10, bed rest day 10.

Table S2 Biometric data of the cancer patient cohort

Subject ID	classification	Weight Loss %	Tumor site	Age	Sex
C002	ctr	0	na	74	F
C003	ctr	0	na	56	F
C004	ctr	0	na	81	F
C010	ctr	0	na	52	F
C012	ctr	0	na	49	M
C015	ctr	0	na	75	F
C020	ctr	0	na	44	F
C021	ctr	0	na	40	M
C022	ctr	0	na	81	M
C024	ctr	0	na	75	F
C080	ctr	0	na	75	F
C107	ctr	0	na	46	F
KC31	ctr	0	na	61	M
KC37	ctr	0	na	61	M
K091	C	17,54	PANCREAS	69	F
K092	PC	0	CRC	42	M
K093	C	10,9	AMPULLA WATER	61	M
K094	C	8,33	PANCREAS	70	F
K095	PC	0	PANCREAS	74	F
K096	C	14,93	PANCREAS	72	F
K101	PC	4,69	PANCREAS	71	F
K104	C	12,96	AMPULLA WATER	77	F
K106	PC	0	PANCREAS	67	F
K108	PC	0	CRC	73	F
K110	PC	0	BILE DUCT	62	M
K112	C	7,78	PANCREAS	70	M
K114	PC	0	BILE DUCT	54	M
K117	C	15,15	PANCREAS	61	M

C, cachexia; PC, pre-cachexia.

Supplementary datasets legends

Table S3. Proteomic data for bed rest cohort. Data are LFQ intensity Log2.

Table S4. Proteomic data for cancer patients. Data are LFQ intensity Log2.

Table S5. Significant hits from paired t-test significant. Proteins significant in both comparisons are highlighted in yellow

Supplementary figures

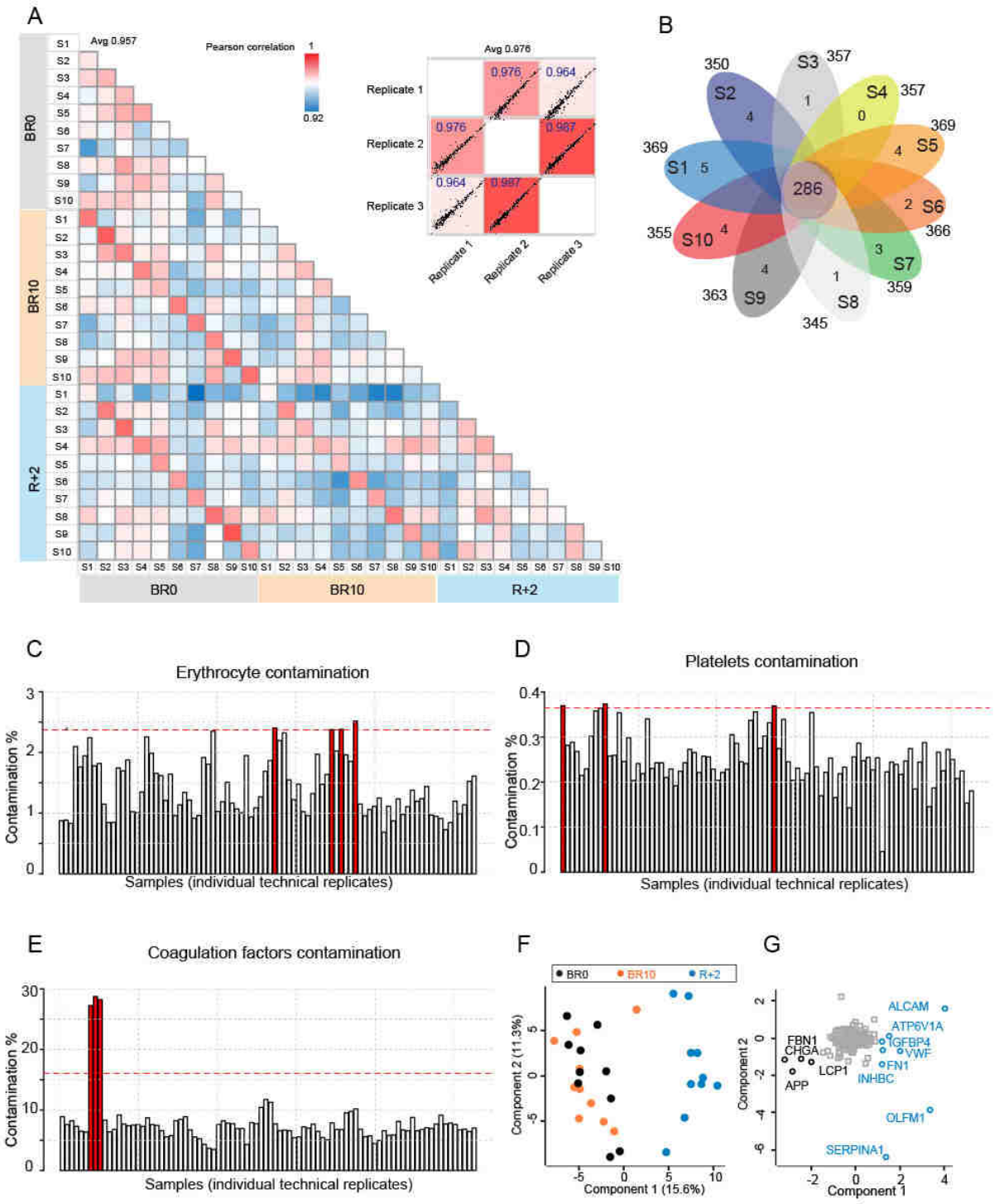

Figure S1 Proteomic features of the plasma bed rest dataset

(A) Matrix of correlations between label-free quantification (LFQ) intensities of ten subjects (median of technical replicates, N=10 per data point). Right panel, same analysis for the median of each timepoint in the three technical replicates. Color scale on top. (B) Venn diagram showing the number of common proteins in the bed rest dataset (three time points and ten study subjects) and of proteins exclusively quantified in one subject. The total number of proteins quantified in each subject is listed outside the corresponding area (C) Bar graph showing percent of contamination of the plasma proteome by erythrocytes-derived proteins as a result of hemolysis. (D) As in C, contamination by platelets-derived proteins presumably due to partial coagulation-induced activation. (E) As in C for coagulation factors contamination, possibly caused by inefficient mixing of the anticoagulant with the blood (here occurring in only one sample, shown in technical triplicates). (F) Principal Component Analysis (PCA) separating the plasma proteome of ten subjects at BR0 (black) and BR10 (orange) from R+2 (blue), suggesting that reloading after bed rest causes extensive plasma proteome remodeling. (E) PCA loadings, with the proteins driving the separation between the groups labelled in the corresponding colors.

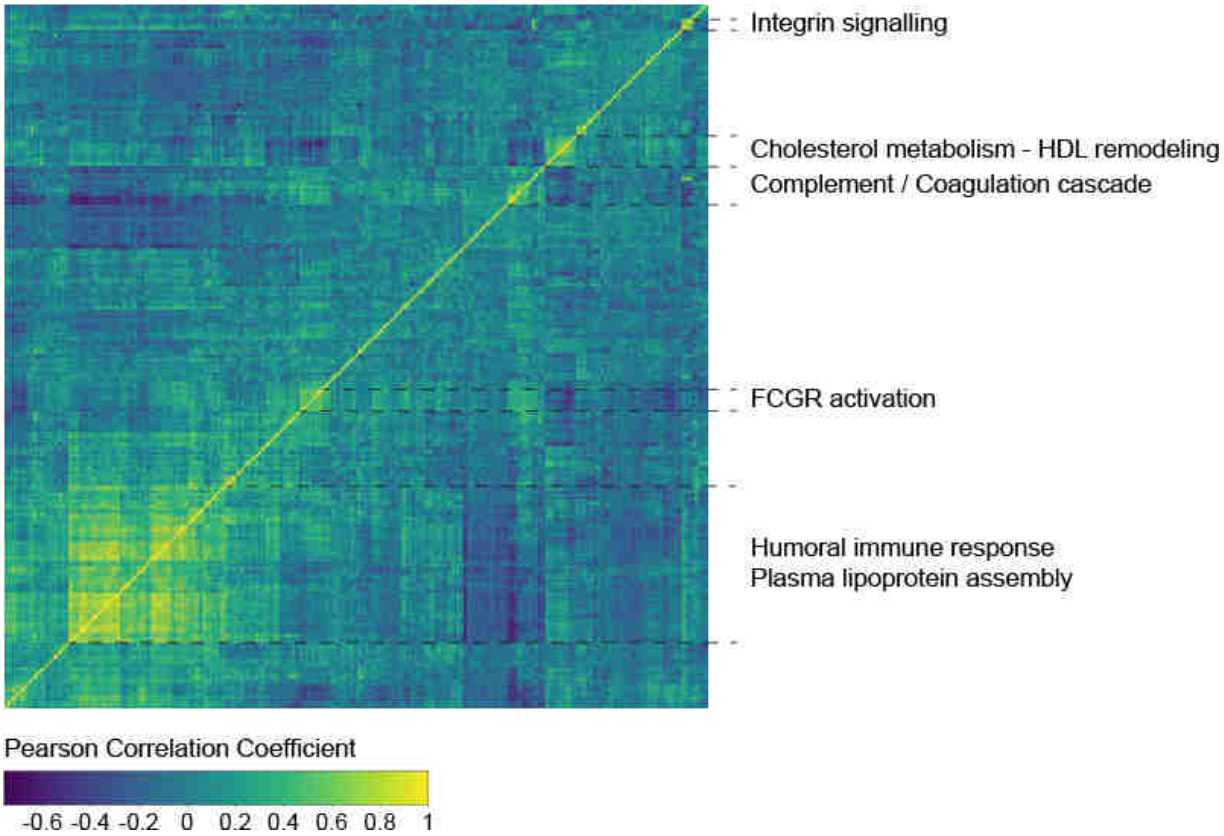

Figure S2 Protein–protein correlation map of bed rest plasma proteome

Matrix of pairwise correlation coefficients where each protein is compared to all others. Proteins with a high positive correlation with each other will cluster together in groups of yellow rectangles (Pearson correlation >0.8). Negative correlation is indicated in blue patches (Pearson correlation <0)

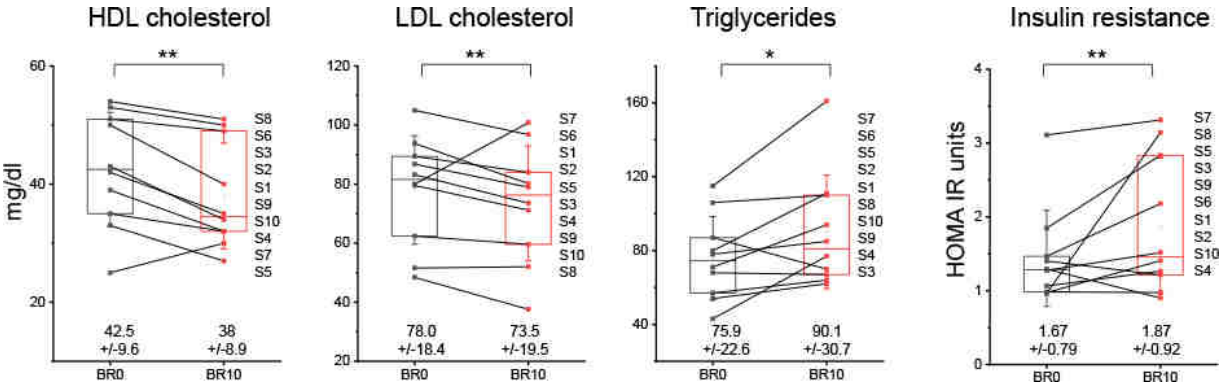

Figure S3 Lipid profile and insulin sensitivity index of the ten subjects before and after bed rest

Variation of plasma concentration (mg/dl) between BR0 and BR0 in each subject (see list next to the plot) for HDL cholesterol, LDL cholesterol and triglycerides. On the right, variation of HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), a readout for insulin resistance, see supporting methods. Box shows median, mean, 75th and 25th percentile, whiskers show standard deviation. *, p<0.05, **, p<0.01 paired t-test, N=10. Median values for all subjects +/- standard deviation are shown at the base of the graphs

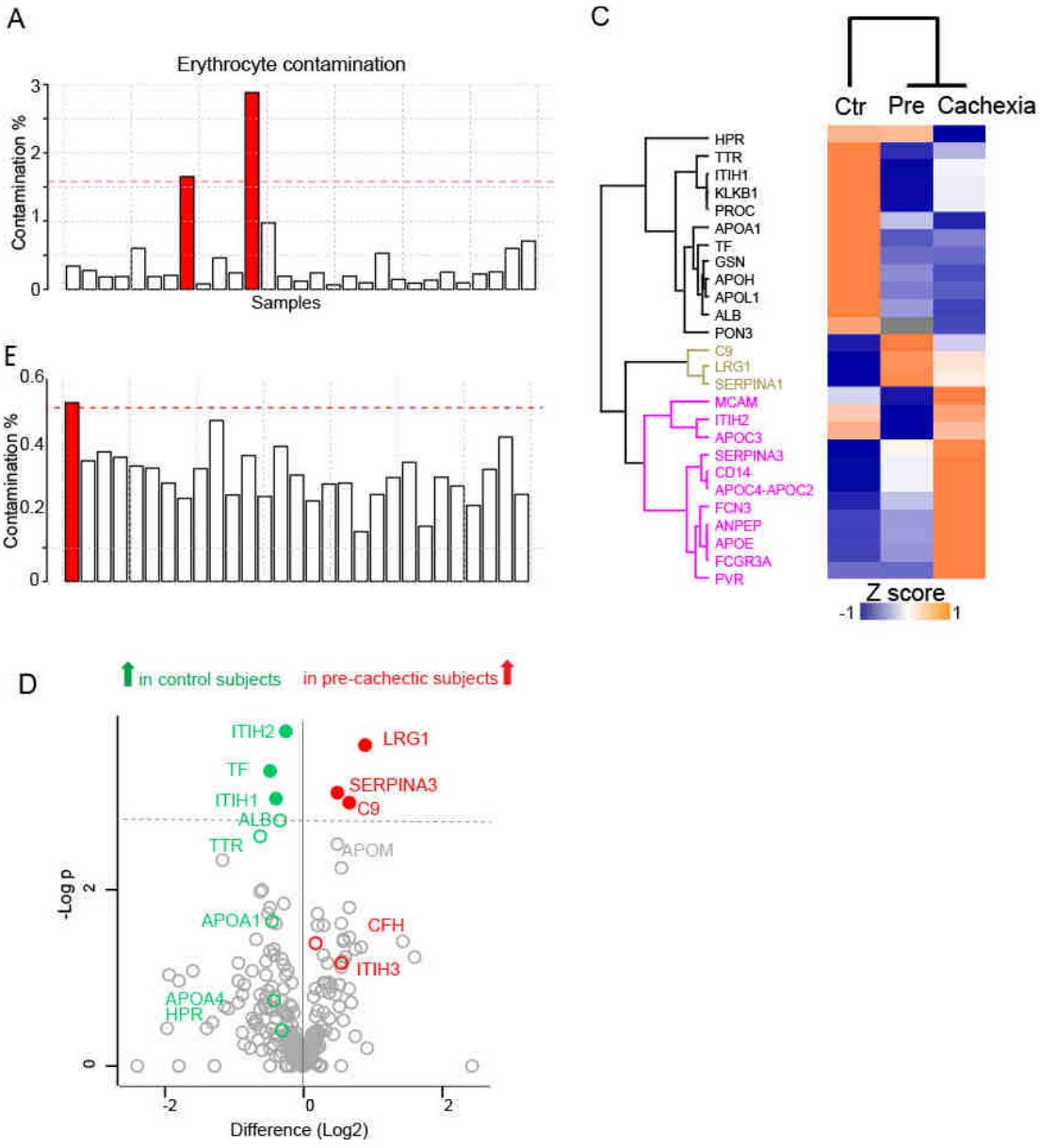

Figure S4 Cancer patients and age-matched controls

(A) Bar graph showing minimal contamination of the serum proteome by erythrocytes-derived proteins as a result of hemolysis. (B) Bar graph showing percent of contamination by platelets-derived proteins. (C) Unsupervised hierarchical clustering of proteins with significantly different expression ($p<0.05$) between at least two of the groups of subjects classified as control (N=14), pre-cachectic (N=7) and cachectic (N=7). ANOVA with Tukey's HSD post-hoc tests. (D) Volcano plot comparing the serum proteome of control and pre-cachectic subjects. Filled dots, proteins with statistically significant serum abundance difference ($p<0.05$, dashed grey line). Empty dots highlighted in red and green, proteins resulting significantly different comparing cachectic subjects with controls