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200+ Protein Concentrations in Healthy Human Blood Plasma: Targeted Quantitative SRM SIS Screening of Chromosomes 18, 13, Y, and the Mitochondrial Chromosome Encoded Proteome

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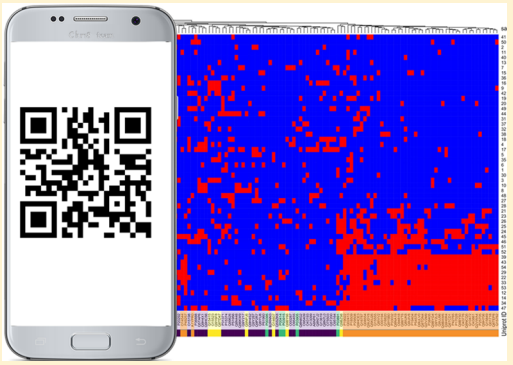
Supporting Information

ABSTRACT: This work continues the series of the quantitative measurements of the proteins encoded by different chromosomes in the blood plasma of a healthy person. Selected Reaction Monitoring with Stable Isotope-labeled peptide Standards (SRM SIS) and a gene-centric approach, which is the basis for the implementation of the international Chromosome-centric Human Proteome Project (C-HPP), were applied for the quantitative measurement of proteins in human blood plasma. Analyses were carried out in the frame of C-HPP for each protein-coding gene of the four human chromosomes: 18, 13, Y, and mitochondrial. Concentrations of proteins encoded by 667 genes were measured in 54 blood plasma samples of the volunteers, whose health conditions were consistent with requirements for astronauts. The gene list included 276, 329, 47, and 15 genes of chromosomes 18, 13, Y, and the mitochondrial chromosome, respectively. This paper does not make claims about the detection of missing proteins. Only 205 proteins (30.7%) were detected in the samples. Of them, 84, 106, 10, and 5 belonged to chromosomes 18, 13, and Y and the mitochondrial chromosome, respectively. Each detected protein was found in at least one of the samples analyzed. The SRM SIS raw data are available in the ProteomeXchange repository (PXD004374, PASS01192).



KEYWORDS: *plasma proteome, targeted proteomics, selected reaction monitoring (SRM SIS), healthy human, Chromosome-centric Human Proteome Project (C-HPP), Human Plasma Proteome Project (HPPP)*

INTRODUCTION

Within the framework of the international Chromosome-Centric Human Proteome Project (C-HPP), the Russian Consortium is developing plasma analysis technology using the double targeted strategy, which combines the chromosome-centric approach with bottom-up Selected Reaction Monitoring with Stable Isotope-labeled peptide Standards (SRM SIS).[1,2] The use of this technology has a significant advantage over alternative approaches because it allows the collection of data on the protein concentrations in the blood plasma with the greatest sensitivity.[3]

The 100% coverage of the protein sequence using bottom-up MS is not attainable; thus, it is impossible to detect all

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Table 1. Baseline Metrics and Meta-Analysis Data for Human Chromosomes 18, 13, Y, and MT (April 2018)

Chr	PCG	PE1 genes[a]	"missing" protein (PE2 + PE3 + PE4)	PRIDE (shotgun LC−MS/MS)	Human Plasma PeptideAtlas	PASSEL (SRM)	Plasma Proteome DB (PPDB)
18	266	248	18	270	52	33	22
13	322	292	25	319	50	10	16
Y	40	27	13	37	0	0	0
Mt	15	15	0	13	1	0	0
total	643	582	56	639	103	43	38

[a]The number of protein-coding genes (PCG, neXtProt, March 2018 release), for which the presence of protein was observed at the proteome level (PE1); "*missing proteins*"—there is no evidence of protein expression; PRIDE[35]—PCG, for which the presence of protein was confirmed using panoramic methods (shotgun LC−MS/MS, only data for human blood plasma are presented); Human Plasma PeptideAtlas[36]—PCG, for which the presence of protein in human blood plasma was confirmed with confidence using panoramic methods; PASSEL[37] (the PeptideAtlas SRM Experiment Library)—PCG, for which the presence of protein was confirmed (SRM, only data for human blood plasma are presented); PlasmaProteomeDB[38]—PCG, for which the presence of protein was confirmed using shotgun LC−MS/MS label-free quantification (spectral counting[39]) and ELISA.[40]

proteoforms expressed from the same gene. Generally, proteome investigations are focused on the master proteins resembling at least one of the many possible proteoforms coded by the gene and containing at least one MS-detectable proteotypic peptide. The sequence can be modified or non-modified, which means that the master protein can be present as a single protein or as a group of proteins. Master proteome of a single chromosome is the result of the identification and measurement of all master proteins encoded by the chromosome and expressed in the selected type of biological material.[4] The objective of this work was to quantify *master proteins*[4] (herein after, the proteins) encoded by chromosomes 18 (Chr 18, selected for C-HPP[5] by the Russian proteomic consortium), 13 (Chr 13, South Korea), and Y (Chr Y, Iran) and the mitochondrial chromosome (Chr MT, Italy) in human blood plasma using a gene-centric approach. For the study, 54 medically well-examined volunteers were selected whose health conditions were consistent with requirements for astronauts.[3,6,7] The study aimed to measure the proteomic profiles and decipher the normal range of protein concentrations in the blood plasma of healthy people.

Although the study of blood plasma had become an independent international initiative (HPPP, Human Plasma Proteome Project[8]), at the moment, there is not much information available in specific resources on the concentration of blood plasma proteins (see Table 1 representing the results of a meta-analysis). Results of text-mining analysis of the summary of scientific publications in the PubMed[9] library show that, regardless of the disease condition, the same group of proteins can be identified as differentially expressed in blood plasma.[10] This is probably due to sensitivity limitations of the proteomic methods used, which do not allow the detection of low-copy-number proteins.[11] Another assumption is that different proteoforms[12] encoded by the same gene are detected in the experiment, but analytical "*myopia*"[13] do not allow one to see them distinctly and, especially, analyze them in quantitative mode.

Chromosomes selected for analysis contained a total of 667 protein-coding genes (PCGs), however 24 of them have PE5 ("Uncertain") status according to neXtProt. PE1-PE4 PCGs were analized during the study. For 91% of them, the presence of the corresponding protein has been confirmed, that is, the corresponding record in the neXtProt[14] database has the status of "*Protein evidence 1: evidence at protein level*". Database information on the presence of the protein was mainly obtained using shotgun LC−MS/MS methods. PRIDE contains mass spectra that confirm the identification of 639 proteins from those 643 protein-coding genes. According to PRIDE, not all proteins identified by shotgun proteomics have the status of "*Protein evidence 1: evidence at protein level*". A discrepancy between these data is related to requirements for the quality of the interpretation of the experimental results established by C-HPP.[15]

Concurrently, SRM technology allowed the detection of the presence of only 43 proteins from the list of interest (see PASSEL) due to the higher labor- and cost-intensity of the SRM SIS workflow, which includes a selection of specific peptides, synthesis of isotope-labeled peptide standards, and the data analysis, among others.[16] However, the SRM SIS method, in contrast to shotgun LC−MS/MS, is better as a quantitative method, which makes it more suitable for practical applications such as medical uses.[17,18]

This study was aimed to quantitatively analyze the proteins encoded by 643 genes in the blood plasma of healthy, clinically well-examined people using SRM SIS technologies.[1] The HPP guidelines 2.1 include the use of two peptides per protein for shotgun technology. At the same time it does not include any requirements for protein measuring using the SRM technology. Our approach involves using SRM technology both for identification and quantitative measurements.

MATERIALS AND METHODS

Subjects

At the Institute of Medico-Biological Problems Russian Academy of Science (Moscow, Russia), 54 male subjects (age 20−47, average 26) were examined by the medical evaluation board, which specializes in space biology and medicine.[19] According to their health characteristics, all the participants were approved for space-related simulations and experiments. They were HIV and viral hepatitis B and C negative and had no previous history of cancer. Routine biochemical and blood parameters of these volunteers were measured using standard automatic analyzers. Most of the parameter values fitted the normal intervals known in clinical laboratory practice (see Table S3, Supporting Information).

All participants provided informed consent to participate in this study. Human-related procedures were performed at the Institute of Medico-Biological Problems according to the guidelines of the local ethical committees.

Sample Preparation for SRM SIS

Venous blood was collected from the volunteers into EDTA Vacutainer plasma tubes (BD). The blood samples were processed according to the manufacturer's instructions. The

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plasma supernatant was filtered through 0.22 μm cellulose-acetate filters (Whatman) and stored at −80 °C. The plasma samples obtained were depleted using a MARS (Multi-Affinity Removal System) Hu-14 column (10 × 100 mm) according to the manufacturer's protocol (Agilent). The collected fractions containing unbound proteins were desalted using cellulose-acetate 5K MWCO (Agilent) spin columns and concentrated to a final volume of 50 μL. The protein concentration was determined using the Micro BCA protein assay (Thermo Scientific, Rockford, IL).

A plasma sample amount of 100 μg (typically 2−2.5 μL) was transferred into a clean tube, and denaturation solution (5 M urea, 1% deoxycholic acid sodium salt, 15% acetonitrile, 100 mM phosphate buffer pH 6.3, 300 mM sodium chloride, 20 mM TCEP) in a volume of 20 μL was added to the sample to a final concentration of total protein close to 10 mg/mL. Samples were heated for 10 min at 60 °C, cooled at room temperature, and supplemented with 25 μL of 15 mM 2-iodoacetamide in 50 mM triethylammonium bicarbonate. The alkylation reaction was incubated for 30 min at ambient temperature following dilution up to 120 μL in 50 mM triethylammonium bicarbonate to decrease the final concentration of denaturation buffer compounds and achieve a final protein concentration close to 1 mg/mL. Trypsin in the amount of 1 μg (5 μL of 200 ng/mL) was added to the samples and incubated for 3 h at 38 °C, following the addition of the next aliquot of enzyme (1 μg) and incubation for an additional 3 h. After the completion of digestion, 10 μL of 10% formic acid was added to the samples. The samples were centrifuged for 10 min at 10 °C at 12 000*g* to sediment the deoxycholic acid. The supernatant was quantitatively (90 μL) transferred into a clean tube and fortified with 10 μL of internal standard solution.

Proteotypic Tryptic Peptide Selection

Manual selection of the unique proteotypic peptides and the most intense transitions was performed on the basis of SRM SIS scouting of Chr 18,[3] Chr 13[7] results, and bioinformatics analysis of proteomic repositories (PRIDE, ProteinAtlas, PeptideAtlas, SRMatlas) and using the neXtProt peptide uniqueness checker[20] as well. Briefly, the desired peptides should be unique across all human genes; the peptide should have a sequence longer than eight amino acids, and some chemically labile amino acids (M, C) should be avoided. We obtained prototypic peptides for 619 proteins (one peptide per protein). The routine method development of SRM comprised several steps, as presented in the Supporting Information and described in detail in Kopylov et al.[16]

Internal Standards

The desired peptides were produced using solid-phase peptide synthesis on the Overture (Protein Technologies) or Hamilton Microlab STAR devices according to the published method.[21] Isotope-labeled leucine (Fmoc-Leu-OH-13C6,15N), arginine (13C6,15N4), lysine (13C6,15N2), or serine (13C3,15N1) were used for peptide synthesis instead of the unlabeled leucine, arginine, lysine, or serine, respectively. The concentrations of the synthesized peptides were measured by amino acid analysis with fluorescent signal detection of amino acids derived after peptide acidic hydrolysis.[22]

SRM SIS Analysis

The separation of peptides from digested plasma was carried out using the HPLC Agilent 1290 system, including a pump and an autosampler. The sample was loaded into the analytical column Eclipse Plus SBC-18, 2.1 × 100 mm, 1.8 μm, 100 A. Peptide elution was performed by applying a mixture of solvents A and B. Solvent A was HPLC-grade water with 0.1% (v/v) formic acid, and solvent B was 80% (v/v) HPLC-grade acetonitrile/water with 0.1% (v/v) formic acid. The separations were performed by applying a linear gradient from 3% to 32% solvent B over 50 min and then from 32% to 53% solvent B over 3 min at 300 μL/min, followed by a washing step (5 min at 90% solvent B) and an equilibration step (5 min at 3% solvent B). Portions of 10 μL of each sample were applied to a chromatographic column. The quantitative analysis was performed using an Agilent 6495 triple quadrupole instrument (Agilent) equipped with the jet stream ionization source. The following parameters were used for the Agilent jet stream ionization source: temperature of the drying gas 280 °C, pressure in the nebulizer 18 psi, flow of the drying gas 14 L/min, and voltage on the capillary 3000 V. The samples were supplemented with 50 μL of 30% formic acid containing all isotope-labeled standards at various concentrations and centrifuged at 10 000*g* for 10 min. Pass-through peptide fractions were used for further SRM analysis.

Data Processing

For each protein, one standard peptide with three transitions was used. The peptides selected were arranged into SRM SIS assays. Information (m/z of precursors, m/z of transition ions, CE values, b- and y-transition ions, and MS platform used for the analysis) on SIS and the target peptides is provided in Table S4, Supporting Information.

Each SRM SIS experiment was repeated in three technical runs. The results were verified using Skyline 4/1 software[23] to identify transitions that were similar to those in the target peptides. For interference screening, we applied the criteria described in Percy at al.[24] Briefly, the peptide was considered to be detected in the run if the differences between relative intensities for three transitions of endogenous and isotopically labeled peptide did not exceed 25% in the run, and the transition chromatographic profiles of endogenous peptide were identical to the corresponding transitions of the stable-isotope-labeled standard.

Calibration curves were obtained for each of the desired peptides using the mixtures of purified synthetic native peptides in a concentration range from 10^{-8} to 10^{-13} M, and their isotopically labeled counterparts were added at a concentration from 10^{-9} to 10^{-12} M. All calibration curves were linear in the range from 10^{-9} to 10^{-13} M and showed a coefficient of linear regression equal to 0.95.

The performance of the SRM SIS used was validated by obtaining the calibration curves of the corresponding set of SIS and synthetic natural peptides. After five SRM SIS runs, we verified the relevance of calibration by analyzing one of the calibration peptide solutions at 10^{-10} M.

The detection limit was defined as the lowest concentration determined in the linear part of the calibration curve. It varied for different peptides in the range from 10^{-13} to 10^{-11} M.

Labeled/unlabeled peptide peak area ratios were used to calculate the concentration of the targeted peptide in a sample.

$$C_{pept} = C_{lab} \times S_{pept}/S_{lab}$$

Here, C_{pept} is the target peptide concentration, C_{lab} the labeled peptide concentration, S_{pept} the area of target peptide peak, and S_{lab} the area of labeled peptide peak.

Statistical analysis and visualization were performed using R software (www.r-project.org). Heat maps were generated via the

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Table 2. Proteins of Chromosomes 18, 13, Y, and MT in the Human Blood Plasma of a Healthy Person

Chr	genes	peptide standards synthesized	whole plasma[a]	depleted plasma	combined data	intersection
18	266	261	56 (25)	59 (28)	84	31
13	322	315	76 (38)	68 (30)	106	38
Y	40	32	10 (0)	10 (0)	10	10
Mt	15	11	5 (0)	5 (0)	5	5
total	643	619	147 (63)	142 (58)	205	84

[a]The amount of PCG for which the proteins were only found in a specific type of biomaterial is indicated in parentheses.

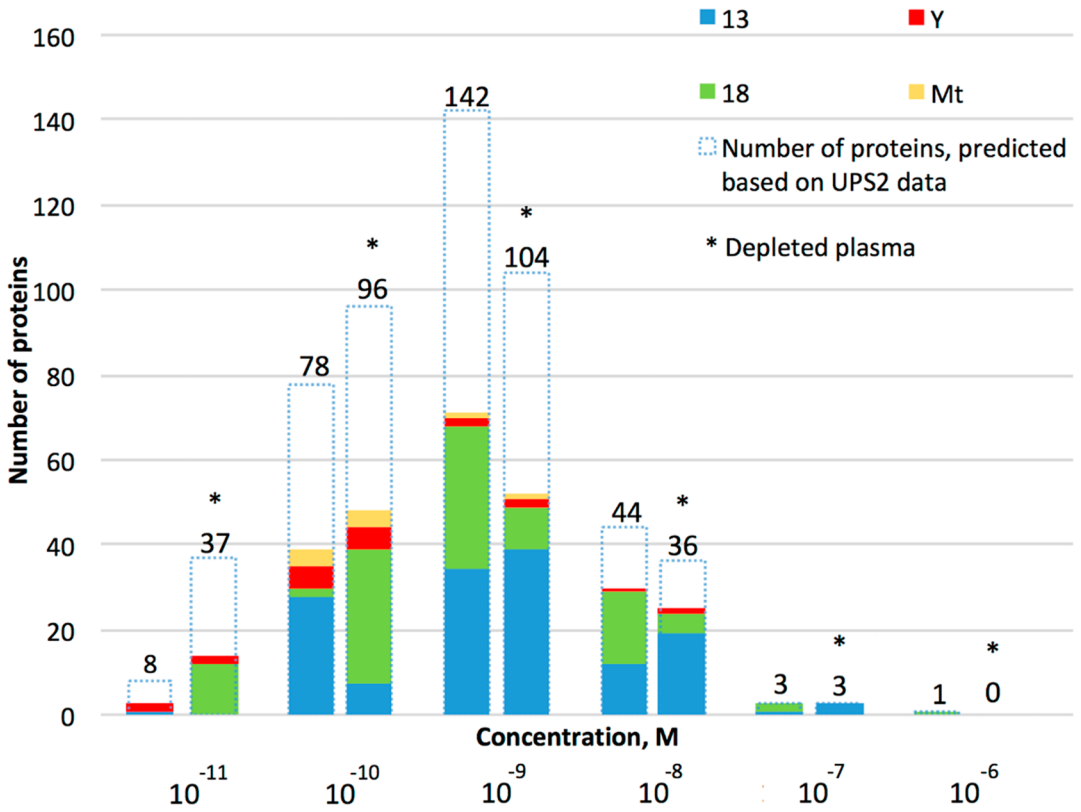

Figure 1. Distribution of the concentrations of the proteins encoded by chromosomes 18, 13, Y, and MT in whole (n = 147) and depleted (n = 142) blood plasma of a healthy person (average data for all samples, n = 54). A dotted line indicates the theoretically possible number of proteins calculated on the basis of the UPS2 model set.[30]

ComplexHeatmap package.[25] Protein dendrograms were built using complete linkage hierarchical clustering.

Over-representation analysis of Gene Ontology biological processes was performed and visualized using the clusterProfiler library.[26] Obtained p-values were adjusted according to the Benjamini–Hochberg procedure (www.jstor.org/stable/2346101).

For the cluster of samples enriched with Chr 18 proteins (see the Results and Discussion section), we evaluated differences in means for 73 standard blood biochemical assays via the Wilcoxon rank sum test. Assays with a p-value less than 0.05 were considered significantly different between samples enriched with Chr 18 proteins and the remaining samples.

RESULTS AND DISCUSSION

Quantitative measurements of blood plasma proteins were carried out for proteins encoded by several human chromosomes (Chr 18, 13, Y, and MT). Methods of targeted mass-spectrometric measurement of protein concentration were scheduled to be developed for 643 protein-coding genes (PCGs) of the above-mentioned chromosomes. This number corresponded to 3% of the total number of human PE1-PE4 PCGs according to neXtProt v2.14.0, data release 2018-01-17.

From all PCGs selected for analysis, it was possible to establish a method for quantitative measurements for 619 PCG only (96% of the total number of PCGs, see Table 2). For other genes (see Table S5, Supporting Information), it was not possible to select a proteotypic peptide for synthesis: no unique proteotypic peptides or peptides which did not match the selection criteria.[16] The numbers of detected and quantified proteins were similar in whole (147) and depleted (142) plasma (see Figure S1, Supporting Information, for Venn diagram). Combining the results for whole and depleted samples allowed us to increase the number of quantified proteins up to 30% of its original number. In total, the products of 205 PCGs were detected and quantified in at least one of 54 whole or depleted blood plasma samples (see Tables S6 and S7, Supporting Information). It is likely that the same protein cannot always be found in all samples because protein concentrations within the proteome profile vary greatly, preventing the identification of a

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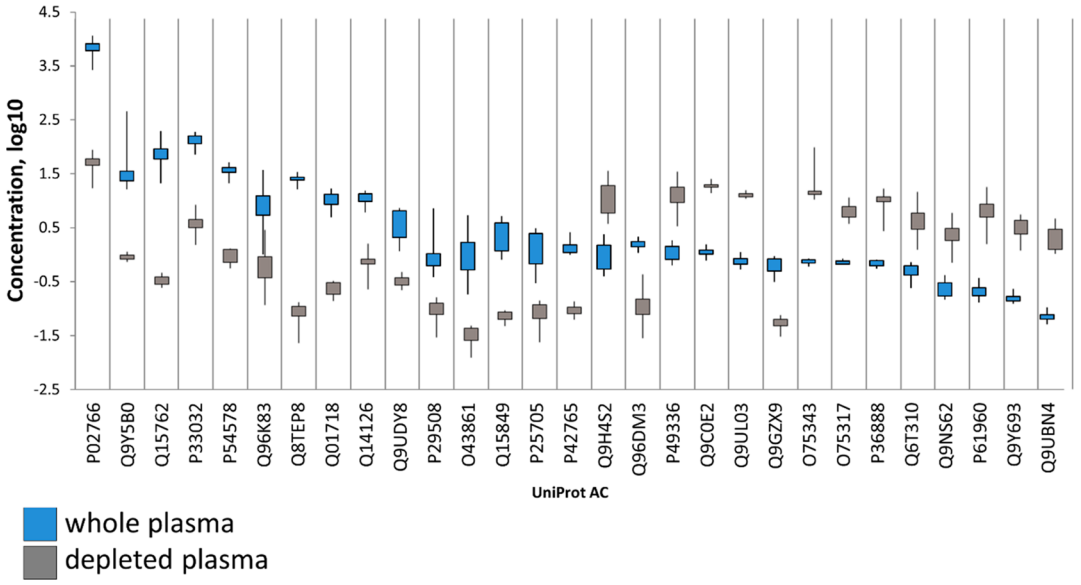

Figure 2. Protein concentrations (log10(copies) in whole and depleted blood plasma.

Table 3. Protein Distribution by Frequency of Occurrence in the Samples

		number of samples in which the protein was detected by SRM SIS		
type of biomaterial	chromosome #	frequently, ≥75% ($n \geq 41$)	25–75%	rarely, ≤25% ($n \leq 13$)
depleted blood plasma	number of proteins	18	62	62
	Chr 13	10	23	35
	Chr 18	8	35	16
	MT	0	1	4
	Y	0	3	7
whole blood plasma	number of proteins	20	70	57
	Chr 13	8	32	36
	Chr 18	12	34	10
	MT	0	1	4
	Y	0	3	7

specific proteotypic peptide due to the limited sensitivity of the technology.[11]

The ratio of the number of proteins detected to the total number of protein-coding genes was also constant if we referred to results obtained in chromosome-centered mode. Thus, proteins corresponding to 32% of the PCG were measured for Chr 18, 33% for Chr 13, and 33% for Chr MT. A proportion of proteins of sex Chr Y was significantly smaller, such that products were found for 25% of the PCG. As expected, the sex chromosome was translated much less extensively compared with somatic ones.[27] One possible way to increase the sensitivity of the SRM SIS method is using two-dimensional separation of peptides using the RP-RP-HPLC system.[28]

The distribution of proteins by concentration in whole and depleted blood plasma is shown in Figure 1. The figure shows that the concentration range, covered by SRM SIS, was 6 orders of magnitude in whole plasma and 5 orders of magnitude in depleted samples. The maximum number of the proteins measured was represented in the 10^{-9} M range. The most abundant protein among all the analyzed products was transthyretin (P02766) encoded by Chr 18. Its concentration in whole blood plasma was 7.1×10^{-6} M. In contrast, the least abundant proteins were found at concentrations of 10^{-11} M, such as three proteins (Q9NPJ6, Q9Y6F8, and Q9UBN4) in whole blood and 14 in depleted samples (see Tables S6 and S7, Supporting Information).

Figure 1 shows the distribution of the concentrations of proteins in the human blood plasma for each of the coding chromosomes and the sample preparation—whole or depleted (marked with *). Proteins encoded by chromosomes Y and MT,

Table 4. Average Concentration of Frequently Detected Proteins

			whole plasma		depleted plasma	
entry	protein names	gene name	number of samples	average concentration, M	number of samples	average concentration, M
P02766	transthyretin	TTR	54	7.1×10^{-6}	54	6.2×10^{-8}
P00742	coagulation factor X	F10	54	1.7×10^{-7}	54	1.8×10^{-7}
P08709	coagulation factor VII	F7	54	1.9×10^{-8}	54	1.8×10^{-8}
P11279	lysosome-associated membrane glycoprotein 1	LAMP1	54	9.5×10^{-9}	54	9.7×10^{-9}
Q14126	desmoglein-2	DSG2	48	1.1×10^{-8}	53	8.2×10^{-10}
Q9Y2J2	band 4.1-like protein 3	EPB41L3	47	2.5×10^{-7}	53	3.4×10^{-8}
Q96IY4	carboxypeptidase B2	CPB2	54	7.5×10^{-11}	53	2.3×10^{-9}
Q06136	3-ketodihydrosphingosine reductase	KDSR	52	8.9×10^{-8}	52	6.0×10^{-8}

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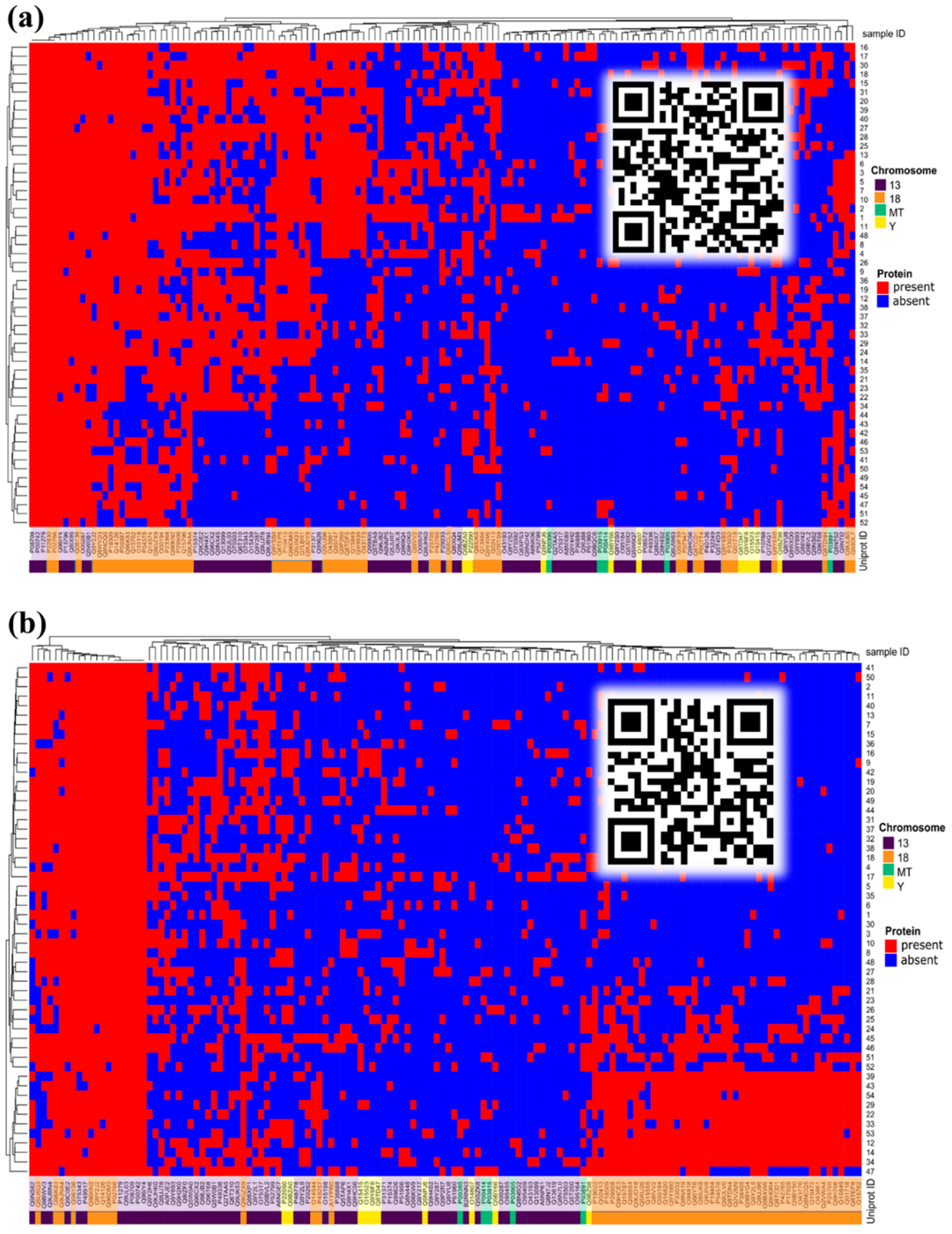

Figure 3. Results of the study of (a) whole and (b) depleted human blood plasma (see description in the text). The UniProtID of proteins are indicated horizontally (the chromosome encoding the corresponding protein is marked with a color); the sample code of the biological material is indicated vertically. If the protein was detected in the sample, the cell was highlighted in red. Blue indicates that the protein was not detected in the particular sample. A QR-code for access to the Supplementary Table is provided.

which demonstrate a significant difference from other human chromosomes,[27] were mainly present in the range of high and medium concentrations (ranges of "proteomic iceberg":[29] high, $\geq 10^{-8}$ M; medium, 10^{-8}–10^{-12} M; low, 10^{-12}–10^{-16} M; ultralow, 10^{-16}–10^{-18} M). It is noteworthy that, for Chr 13 in the 10^{-10} M range, the most number of proteins was detected in whole plasma, while the situation was different for Chr 18, with most proteins detected at a concentration of 10^{-9} M. The difference between adjacent orders of magnitude was not a very reliable boundary and can be explained by the allowed error of quantification. Thus, Figure 1 depicts common trends and imprecise protein properties.

The results for human proteins were compared with those of the analysis of the standard UPS2 set performed using the SRM SIS method (Sigma-Aldrich, 48 proteins, eight proteins in six concentration ranges from 10^{-11} to 10^{-6} M). The maximum number of proteins from among those present in the standard kit (7 of 8 proteins[30]) was detected in whole blood plasma for the most highly represented proteins (10^{-6}–10^{-7}M, see Figure 1). Only 19 proteins were found in whole blood plasma from those

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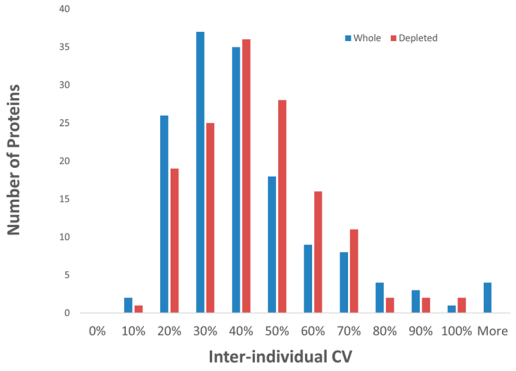

Figure 4. CV distribution histogram.

contained in the kit at a concentration of 10^{-11} M. In the concentration range from 10^{-7} to 10^{-10} M, losses caused by technical limitations and interference ranged from 14 to 24 proteins.

Extrapolating the results, obtained on a standard UPS2 set in the presence of whole blood plasma and extract of *Escherichia coli*,[30] to the proteins encoded by chromosomes 13, 18, Y, and MT, it can be assumed that, for example, at levels of 10^{-11} M, approximately 60% of the total number of proteins remained undetected (see Figure 1). Summarizing all the concentration ranges, the potential number of proteins measured in the experiment with whole plasma could be 276 rather than 147 (with depleted plasma, 277 instead of 142), assuming that all the proteins present in the samples in the given concentration ranges could be detected and measured.

Notably, the descending part of the histogram in the range 10^{-6}–10^{-7} M indicated a small number of high-copy-number proteins, i.e., only five proteins in total. In this range, the results of the theoretical prediction using UPS2 analysis data coincided with the number of proteins detected experimentally. In Figure 1, columns depicting proteins found in the experiment coincided with shaded columns, showing the estimated number of proteins.

Comparatively, the experimental results obtained for protein detection in the medium-copy-number interval with levels lower than 10^{-8} M differed significantly from the calculated values (see Figure 1). In this range, most proteins were not detected experimentally because of the technical limitations of the analytical method rather than a real absence of these proteins in the selected type of biological material. According to the study UPS2 set, in the concentrations lower than 10^{-8} M, more than 50% of the total number of proteins known to occur in the analyzed sample remain undetected.[30]

The overlapping between the results of the experiment with whole plasma versus depleted plasma in the case of Chr 18 was 31 proteins measured in both types of samples, a total of 84 measured proteins, see Table 2. Considering all 643 protein-coding genes from 13, 18, Y and MT chromosomes, 84 proteins were measured in both whole and depleted plasma. This overlapping also constituted 40% of 205 measured proteins.

The analysis of the quantitative correlation between average concentrations of proteins measured in whole and depleted blood plasma showed the following. For most proteins, protein concentrations differ within the same order of magnitude. Thus, the ratio of the log10 of the proteins averaged over 54 samples in whole plasma to those in depleted plasma was in the range from −1 to 1 for 55 of 84 proteins. The difference in concentrations reached more than 2 orders of magnitude for three proteins (P02766, Q15762, and Q8TEP8), and the levels of these proteins in whole plasma were 2 orders of magnitude higher than in the depleted one (see Figure 2). It was evident that depletion affected the protein concentrations nonlinearly. The proteins were sorted in the order of decreasing concentration in whole plasma. Of note, for 12 proteins, depletion led to an increase in concentration, while the concentration increased by less than an order of magnitude. Apparently, this phenomenon was observed as a consequence of peptide interference.

The data obtained (see Tables S6 and S7, Supporting Information) were analyzed for the detection of proteins in the upper (75th percentile) and lower (25th percentile) quartiles. Thus, proteins detected in more than 75% of the total number of samples (n = 54, i.e., protein detected in ≥41 samples) were considered *frequently identified*; proteins found in less than 25% of the samples (≤13 samples) were considered *rarely identified*. Table 3 shows the values of the distribution of the measurement results between these indicators.

On average, approximately 20 proteins were detected in 41 or more samples, representing approximately 3% of the total number of protein-encoding genes analyzed. Thus, it can be deduced that for all human protein-coding genes an average of approximately 400 proteins can be obtained from the whole genome by the present method by overlapping individual human proteomic profiles. This is most likely the number of proteins that can be detected using the same approach for every healthy person. On the basis of the data presented in Table 3, the ratio of frequently identified proteins to the total number of protein-coding genes remained approximately constant for somatic chromosomes 18 and 13, but not for the Y and mitochondrial chromosomes. None of the proteins encoded by the Y and MT chromosomes were included in the group of frequently identified proteins, which once again confirmed the essential difference between these chromosomes and the other human chromosomes.[27] Such evidence is particularly interesting since it may open a route for testing peripheral blood mitochondrial heterogeneities, such as those associated with different levels of heteroplasmy in genetic syndromes.

Eight proteins (P02766, P00742, P08709, P11279, Q14126, Q9Y2J2, Q96IY4, and Q06136; see Table 4) were in the group of frequently identified proteins in both whole and depleted blood plasma. Apart from the other group-related proteins, transthyretin (P02766, TTR, Chr 18) showed the most significant concentration inequality between the sample before and after depletion (7.1×10^{-6} M, and 6.2×10^{-8} M, respectively). It can easily be explained by its specific (antibody-mediated) targeting on the Hu-14 column. This result revealed an almost 2 orders of magnitude difference in concentration, which was in complete agreement with the expected protein removal from plasma, although the presence of transthyretin in the postdepleted samples indicated incomplete removal of proteins (which is hardly possible) but emphasizes a sufficient enrichment.

In addition, a list of proteins encoded by Chr 13 and detected in every analyzed sample included P11279 (LAMP1, concentration 9.5×10^{-9} M in the whole plasma, 9.7×10^{-9} M in the depleted), P08709 (coagulation factor VII, concentration 1.9×10^{-9} M in the whole plasma, 1.8×10^{-8} M in the depleted), and P00742 (coagulation factor X, see Table 4 and Tables S6 and S7, Supporting Information). As shown in Table 4, many proteins in the group of frequently identified species were highly abundant proteins. The only exception was Q96IY4 (carboxypeptidase B2) with a concentration in whole blood plasma of 7.5×10^{-11}

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M. Its physical chemistry properties may allow its detection using SRM SIS with a good response.

For an exploration of the relationship between the protein abundance and the number of samples in which this protein was detected, the Pearson correlation coefficient was calculated between the decimal logarithm of the protein concentration and the proportion of samples in which this protein was detected. In this case, the coefficient was $r = 0.46$ for whole blood plasma and $r = 0.28$ for depleted blood plasma (p-value <0.001 in both cases). The obtained correlation value can be considered as low (see the Supporting Information) and lacking biological significance. Thus, it was not possible to confirm relationship between the concentration of proteins in the blood plasma and the number of samples in which these proteins were detected. In our opinion, the concentration of the same protein in humans varied, and because of sensitivity restriction of the analytical method different proteins were detected in the proteomic profile. In other words, differences in the "depth" of the proteome led to differences in "width"[4] due to the "cutoff line" determined by the sensitivity method.

The combined list of frequently identified proteins from the analysis of whole and depleted plasma contained 30 proteins, which were found in at least 41 of 54 samples. A summary of this group of proteins generated using GeneOntology[31] included terms such as *Hemostasis, blood coagulation, Gamma-carboxylation, transport and amino-terminal cleavage of proteins*, and *Cell-cell junction*.

The group of rarely identified proteins was much broader than the group of frequently found species. Combining data on whole and depleted plasma made it possible to group 99 proteins, each of which was found in fewer than 13 out of 54 (≤25%) samples. Thus, the individual proteomic profile could be characterized by a relatively constant part represented mainly by high-copy-number proteins and a variable part represented by a group of proteins that are detected accidentally.

Figure 3 shows a binary data matrix of the study results for whole and depleted blood plasma. In the case of whole blood plasma (Figure 3a), a large number of proteins were found simultaneously in more samples. In the analysis of depleted blood plasma, the number of such proteins in most samples was much smaller (see cluster on the left, Figure 3b). This finding was most likely due to the sample preparation procedure, which involves the removal of high-copy-number proteins. During this procedure, nontargeted proteins can clearly also be removed in an unpredictable manner.[32] In the matrix of depleted plasma there is the cluster of samples for which a relatively large number of proteins encoded by Chr 18 were found (see right lower part, Figure 3b, nos. 12, 14, 29, 33, 34, 39). According to the attached clinical annotations of the samples (see Table S3, Supporting Information), the participants in this group had a significant excess of the level of total and conjugated bilirubin in comparison to the other people studied. The summary of Chr 18 proteins affecting the formation of this cluster of samples provided additional gene ontology terms:[31] "*positive regulation of nucleotide metabolic process*" and "*cAMP biosynthetic process*", likely because this group of proteins can be associated with a bilirubin metabolism disorder.

Interindividual variance was assessed by the concentration of proteins measured in whole and depleted plasma by calculating the coefficient of variance (CV[33]) of values between the analyzed samples. The assessment was carried out in a similar way to the published[3] one: proteins with a CV ≤ 30% were considered to be low-variable, while proteins with a CV ≥ 100% were highly variable.

In whole blood plasma (see Figure 4), 66 of 147 (45%) proteins were characterized by a low CV of the concentration between samples. Of them, 35 proteins were encoded by Chr 13, 25 by Chr 18, 4 by chromosome Chr Y, and 2 by Chr MT. For comparison, the proportion of low-variable proteins in depleted blood plasma was less, 32.4% (46 of 142 proteins), with a p-value calculated by the Fisher exact test of approximately 0.03.

According to the results for the depleted samples, a group of proteins with high variability (more than 100%) was absent, while in whole plasma there were only four such proteins, including Q9Y5B0 (Chr 18), Q53F39 (Chr 18), P29508 (Chr 18), and Q8N0X7 (Chr 13). There was no relationship between the protein CV and its average concentration, or the number of samples in which the protein was detected. At the same time, Hortin et al.[34] showed that 150 high-copy-number proteins also had a high level of variability. We have also previously proposed[3] that proteins present at relatively low concentrations possess a higher level of variability. The present work shows no such relationship in the chromosome-centered mode of analysis.

CONCLUSION

For the practical use of the C-HPP results in medicine, it is necessary to uncover which part of the human plasma proteome could be identified and measured. In the present work we have extended our chromosome-centric studies onto several human chromosomes. By the "*double targeted*" strategy (first target is a chromosome, and second target is the proteotypic peptide for the gene of the chromosome) the concentration for 205 proteins encoded by Chr 18, Chr 13, Chr Y, and Chr MT in blood plasma was accurately measured with SRM-SIS assay. Before this work was carried out, the number of measured in blood plasma proteins encoded by the selected chromosomes was limited to only 38 proteins (according to the meta-analysis presented in the Plasma Proteome DB, see Table 1). Importantly, our measurements were done in the plasma of the volunteers, whose health conditions were consistent with requirements for astronauts. It was shown that the concentration range covered by the SRM SIS technology was 6 orders of magnitude (from 10^{-6} to 10^{-11} M) in the case of the analysis of whole plasma, and 5 orders of magnitude (from 10^{-7} to 10^{-11} M) in the case of the analysis of depleted samples.

We observed that chromosome-centric plasma proteomes were severely susceptible to the depletion of the plasma. Although the laborious step of depletion is hardly imagined as a tool for the clinical proteins in the case of the chromosome-centric approach simultaneous analysis of depleted and nondepleted plasma enabled the gain of an additional 58 of desired proteins.

Unexpectedly, from the obtained data we derived no correlation between protein abundances and corresponding number of samples in which this protein was detected. It might be the result of different factors related to the SRM approach, like lack of unitypic peptides generated by trypsin, signal-to-noise ratio, which depends on the microvicinity for different peptides, etc.

There are only about 30 proteins encoded by the examined 643 protein-coding genes, whose abundance levels are more or less stable (interindividual CV ≤ 20%). It seems that those proteins could be used as a pillar for the creation of SRM-assays for personal health analytics.

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■ ASSOCIATED CONTENT

Supporting Information

Table S3. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00391.

Routine method development of SRM, comparison of whole and depleted human blood plasma, results of the study of whole and depleted human blood plasma (protein concentration matrix), interpretation of *r* value, and estimation of number of detected proteins based on UPS2 set analysis (PDF)

Annotation of human blood plasma samples, list of protein IDs for which no SRM SIS method was developed due to the lack of a suitable proteotypic peptide, whole plasma data, and depleted plasma data (XLSX)

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Author Contributions

¶B.V.M. is deceased since 01.01.2015. He contributed to the development of an optimal method for medical monitoring of the health status of patients using a set of biochemical, immunological, hematological, and hemostasiological indicators applied to the requirements of proteomic analysis.

Notes

The authors declare no competing financial interest.
The SRM SIS raw data are available in the ProteomeXchange repository (PXD004374, PASS01192).

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