

Plasma Fractionation and Mixture Improves Coverage in Proteomic Analysis

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Cite This: <https://doi.org/10.1021/acs.jproteome.5c00814>



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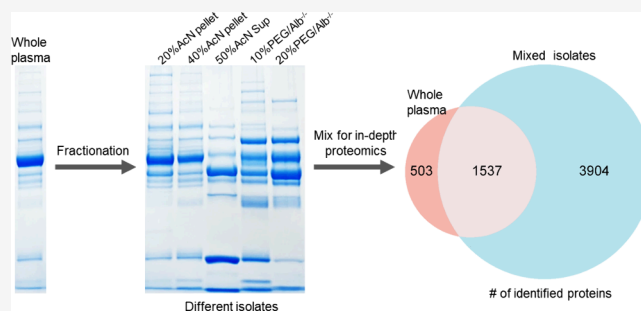
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ABSTRACT: Expanding plasma proteome coverage increases the success in proteomic discovery of blood biomarkers. Here we report that the sequential precipitation of plasma by increasing concentrations of acetonitrile (AcN) can fractionate proteins. Combining some of these fractions with other fractions from polyethylene glycol (PEG) precipitation and albumin depletion to have the mixed sample that included these partitioned fractions: 10% whole plasma, 20% 20%-AcN-pellet, 10% 40%-AcN-pellet, 20% 50%-AcN-supernatant, 20% 10%-PEG-precipitation-albumin-depletion, and 20% 20%-PEG-precipitation-albumin-depletion, has yielded identification of 5441 proteins, remarkably larger than the 2040 proteins detected in the whole plasma directly. This study provides nearly the largest plasma proteomics data sets and recommends this fractionation and mixture strategy as an efficient approach for expanded plasma proteomics coverage.

KEYWORDS: Plasma, proteomics, LC-MS/MS, acetonitrile, polyethylene glycol, albumin depletion, mass spectrometry



INTRODUCTION

The blood plasma is an important reservoir of circulating biomarkers for diagnosis of clinical diseases. Although major advances have been made in technologies like the Proximity Extension Assays (PEA, by Olink) and the SomaScan (by SomaLogic) that can measure thousands of proteins in the plasma,^{1–4} their accuracy remains a concern as specificity of the antibodies or aptamers used in these assays is generally not rigorously validated and verified in clinical settings.^{4,5} The mass spectrometry (MS)-based proteomics is still an essential tool for analyzing proteins in the plasma samples, especially for detecting those not defined in the libraries of PEA and SomaScan kits.

However, limited proteome coverage is a common problem in the MS-based plasma proteomics field. Generally, only around a few thousand proteins were identified in reports and only a few leading groups are able to profile up to 5,000 proteins in plasma or serum samples.^{6–10} Techniques like extensive peptide fractionation,⁸ DMSO in liquid chromatography,¹¹ sophisticated mass spectrometers,¹² and other emerging methodologies^{13,14} have been developed to improve the LC-MS/MS-based plasma proteomics. Although a data-independent acquisition-based SWATH scan can detect extra proteins at very low levels,¹⁵ quantitation of these proteins is challenging, especially in TMT and iTRAQ-based quantitative proteomics where sufficient reporter ions and few coisolated ions are essential. Therefore, the removal of abundant proteins and

extensive peptide fractionation are key factors in quantitative plasma proteomics.

Besides these efforts, plasma preanalysis processing to remove commonly abundant proteins has also been studied, including traditional immunodepletion and recently introduced multi-nanoparticle plasma protein corona method and other modified approaches.^{10,14,16–18} In addition to these, using protein-precipitating reagents to pellet these commonly abundant proteins is also important direction in this field such as trichloroacetic acid.¹⁹ We have found that polyethylene glycol (PEG) can efficiently precipitate immunoglobulins, complements, fibrinogens and other commonly abundant proteins which can thus enrich target proteins efficiently for proteomic analysis.²⁰ Now we find that acetonitrile can also precipitate plasma proteins sequentially at increasing concentrations.

Acetonitrile is an organic solvent and is commonly used in plasma to precipitate proteins for enrichment of low molecular proteins, protein fragments and peptides.^{21–23} It weakens hydrophobic interactions and enhances peptide–peptide hydro-

Received: August 20, 2025

Revised: October 16, 2025

Accepted: October 31, 2025



gen bonds,²⁴ promoting formation of different structural intermediate states of proteins and their significant changes in conformation until denaturation and aggregation at higher concentrations of acetonitrile.^{25–27} When it was used in experiments for this purpose, concentrations higher than 60% were generally used. However, we have found that at lower concentrations starting from 25% to 30%, acetonitrile can precipitate the majority of plasma proteins but exert a much milder precipitating effect on albumin (Supplemental Figure S1). Some proteins such as immunoglobulins, apolipoproteins, and complements were largely precipitated at 10% to 20% acetonitrile, while albumin started to precipitate heavily at 50% acetonitrile (Supplemental Figure S2). This suggests that at certain concentrations, acetonitrile might be able to separate other proteins from albumin for less detection interference in proteomic analysis. If this turns out to be true, combining these acetonitrile-partitioned plasma isolates with our previously developed PEG precipitated- and albumin-depleted plasma fractions together for proteomics analysis might greatly improve plasma proteome coverage.

MATERIALS AND METHODS

Human Plasma Sample Collection

This study used the leftover of the clinical blood samples after clinical tests were completed, and this was approved by the Ethics Committee of our Nanjing Drum Tower Hospital (IRB Review Approval # 2022-165-01). All related analyses abided by the Declaration of Helsinki principles. 100 used human EDTA-anticoagulated blood samples were collected and pooled from patients with different types of diseases or individuals who visited our hospital for health screening (Supplemental Figure S3). Samples with any positive results in immunoassay screening of contagious pathogens (hepatitis B and C, syphilis, and human immunodeficiency virus) were excluded. Each whole blood sample was centrifuged first at 5000g for 5 min and then the supernatant was centrifuged again at 20,000g for 5 min prior to pooling, aliquoting, and storage at 80 °C. The pooled plasma samples received clinical biochemical and protein immunoassays (Supplemental Figures S4 and S5).

Plasma Precipitation by Acetonitrile

The plasma sample was sequentially precipitated stepwise: the supernatant from the precipitation by acetonitrile at a previous concentration was precipitated again by acetonitrile at 10% more concentration. To control the starting plasma volume as the same, the precipitation was actually done like this: six tubes was taken, and 450 μ L of plasma was added to the first tube and 500 μ L to the rest of the five tubes; then 50, 55.6, 125, 214.3, 333.3, 500 μ L of 100% acetonitrile was added to these six tubes respectively to have 10%, 10%, 20%, 30%, 40%, and 50% acetonitrile in plasma; after vortexing and rotation for 15 min at room temperature, the samples were centrifuged. The pellet of the first tube was kept as the 10% acetonitrile precipitation sample; from the rest tubes, 500, 562.5, 642.6, 750, 900 μ L of supernatant (all containing 450 μ L of original whole plasma) were taken, respectively, and 62.5, 80.4, 107.1, 150, and 225 μ L of 100% acetonitrile were to each of these five tubes to increase their acetonitrile by 10%; after vortex, rotation, and then centrifuge, the pellets were taken and cleaned by acetonitrile at a concentration that precipitates them as the sequentially precipitated proteins of 20%, 30%, 40%, 50% and 60%.

Plasma PEG Precipitation and Albumin Depletion

The plasma with 1% Triton X-100 was mixed with 20% or 30% PEG6000 in TBS buffer equal to 10% or 20% for precipitation. After brief vortex and then rotation for 15 min at the room temperature, the mixed samples were centrifuged for collecting the supernatants. The supernatants were loaded to the Cibacron Blue 3G-A agarose column, respectively, and incubated by rotation at the room temperature for 30 min. After that, the column was briefly and gently centrifuged for collection of the flow-through, in which albumin was depleted. Proteins in the final supernatants were precipitated by TCA/acetonitrile for analysis in this study.

LC-MS/MS and the in-Depth Proteomic Analysis

The original whole plasma was precipitated and cleaned with 80% acetonitrile. The precipitated and cleaned protein samples from the plasma and its extracted pellets or supernatants were dissolved in 8 M urea (50 mM triethylammonium bicarbonate buffer, TEAB, pH 8.5) with addition of LysC (1 μ g per 100 μ g proteins) for 4 h at the room temperature until the samples became clear and quantified by the bicinchoninic acid method. After that, trypsin was added (1 μ g per 100 μ g proteins) to start in-solution digestion for 8 h or longer at room temperature, followed by dithiothreitol reduction and iodoacetic acid alkylation and C18 desalting.

The peptide samples were analyzed through a column (50 μ m ID and 30 cm long, packed with C18, 1.9 μ m) under a gradient of acetonitrile for \sim 120 min by the mass spectrometer (Q Exactive HF-X, Thermo) in the data-dependent acquisition mode.

For in-depth analysis, the offline High pH reversed-phase fractionation of the peptides was performed by the C18 column (4.6 mm \times 250 mm, BEH, 3.5 μ m, XBridge) and the high-pressure LC system (Shimadzu LC-20AD with UV detector). Three mg peptides were separated by the linear gradient (first 5% solution B for 10 min, then 5–40% solution B for 100 min, and finally 40–65% solution B for 10 min (A: 10 mM ammonium formate, pH 8.0; B: 90% acetonitrile, 10 mM ammonium formate, pH 8.0) at the flow rate of 1.0 mL/min. About 100 fractions were finally collected and run individually without concatenation, except those beginning or ending fractions.

For the plasma sample partitioning, plasma fractions isolated by acetonitrile, PEG and Cibacron Blue column were digested into peptides and then mixed. Among the six extracts, because 40% acetonitrile has largely precipitated proteins from plasma (Figure 1A,B) and their protein bands are very similar (Figure 1C), the 40% acetonitrile precipitate and the original whole plasma were equally mixed as one sample to mix again equally with the other four extracts with these amounts and the final compositions: 0.5 mg of the original whole plasma (10%), 0.5 mg of 40% acetonitrile precipitate (10%), 1 mg of 20% acetonitrile precipitate (20%), 1 mg of 50% acetonitrile supernatant (20%), 1 mg of 10% PEG-precipitated and albumin-depleted supernatant (20%), and 1 mg of 20% PEG-precipitated and albumin-depleted supernatant (20%). The mixed peptides were extensively fractionated by basic pH RPLC and analyzed by the mass spectrometer, respectively.

The MS raw files were processed by the Proteome Discoverer 2.4 software (ThermoFisher) and the Sequest HT engine with the human proteome database (UP000005640, 83,587 proteins including 20,405 reviewed and 63,182 unreviewed ones) from the Uniprot database. Specific parameters were basically as the

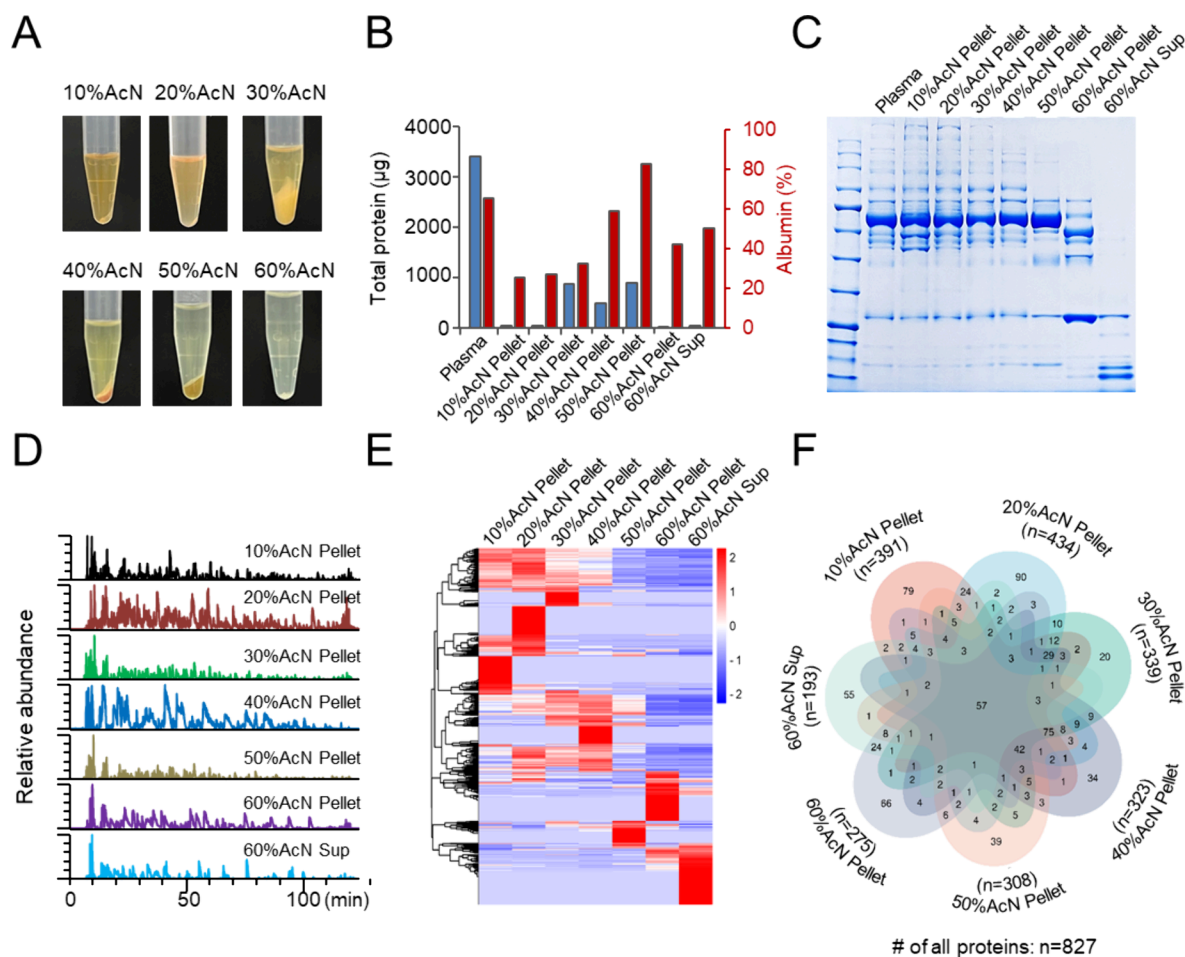


Figure 1. Sequential precipitation of plasma by increasing concentrations of acetonitrile. (A) Plasma precipitated sequentially by increasing concentrations of acetonitrile. (B) Total protein amount and the percentage of albumin in each precipitated pellet. 100 μ L plasma was used. (C) SDS-PAGE and Coomassie blue staining of these pellets. (D) Liquid chromatographs during the LC-MS/MS analyses of these pellets. (E) Heatmap to demonstrate the distinctly enriched subsets of proteins identified in these pellets. (F) Unique and shared proteins identified in these pellets.

default, including full trypsin, maximum cleavages of 2, peptide length ranging from 6 to 144, 10 ppm in precursor mass tolerance and 0.02 Da in fragment mass tolerance, *b* and *y* ions in spectrum matching, maximum modifications of 3 and 15.995 Da (M) with N-terminal acetylation, methionine loss, or both in the dynamic modifications, and carbamidomethyl (+57.021) in the static modifications. Peptide-spectrum matches (PSMs) were verified based on *q*-values at the false discovery rate (FDR) of 1% under the Percolator module. At proteins levels, the Strict and Relaxed FDRs were set at 1% and 5%, respectively. Proteins with *q*-values lower than 1% were assigned with “High” confidence in identification, and those between 1–5% were assigned with “Medium” and those with 5% or higher were assigned with “Low”.

Protein Size, Hydrophobicity, Isoelectric Point, and Domain Analyses

Protein sizes and isoelectric points were downloaded in the searching results from the Uniprot databases (Supplemental Table S1). Protein hydrophobicity is evaluated by the GRAVY (Grand Average of Hydropathy) score which calculates the average of the hydropathy values of all amino acids in a protein (<https://www.gravy-calculator.de/>) with a Python package Biopython 1.83 (Bio.SeqUtils.ProtParam module).²⁸ The domain analysis was performed with the DAVID tool from Pubmed and the InterPro database.²⁹

Data Comparison with Public Databases and Reported Studies

The plasma protein data sets were downloaded from three public databases including Human Plasma Proteome Project Data (<https://peptideatlas.org/hupo/hppp/>),⁴ Human Body Fluid Proteome (HBFP) database (<https://bmbl.bmi.osumc.edu/HBFP/>),³⁰ Human protein Atlas (<https://www.proteinatlas.org/>),^{31,32} and three major plasma proteomics studies.^{7,9,20} Proteins from each data set were all converted to their gene names for combining and listed in our previous report.²⁰ The absolute concentrations of proteins downloaded from the HPA database were converted to nanograms per liter, and the proteins were ranked according to their absolute concentrations from the highest to the lowest.

RESULTS

Plasma Sequential Precipitation by Acetonitrile and Proteomic Analysis

After we found that acetonitrile had a differential precipitating effect on albumin and other proteins (Supplemental Figure S1), we used 10%, 20%, 30%, 40%, 50% and finally 60% acetonitrile to precipitate the plasma sequentially (Figure 1A). Although fewer proteins were precipitated first at 10% and 20% acetonitrile, the percentage of albumin in these two pellets

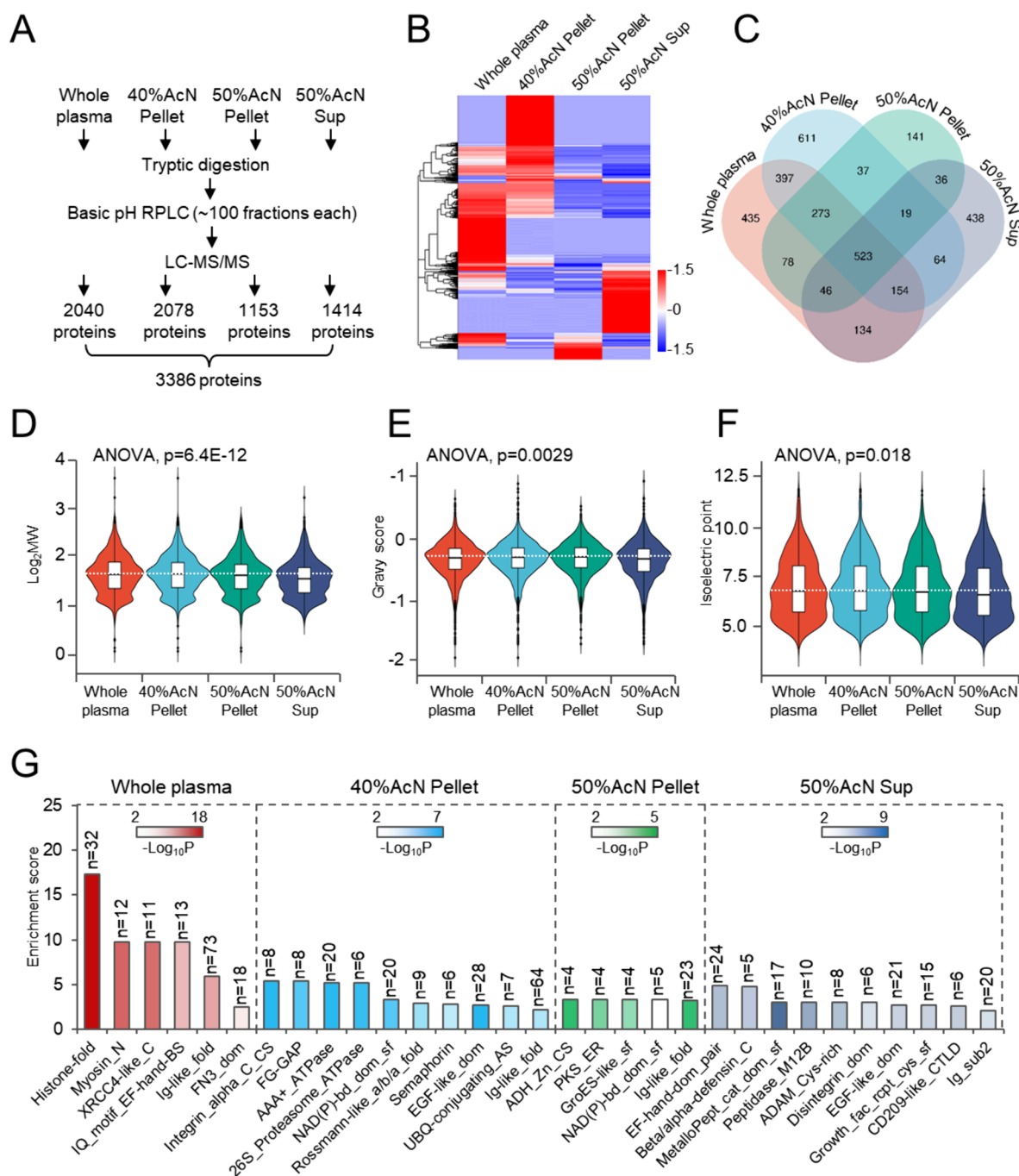


Figure 2. In-depth proteomics analyses of the original whole plasma and the acetonitrile-precipitated pellets. (A) Work flow of the in-depth proteomic analyses. The plasma was precipitated by 40% directly and then further precipitated by 50% acetonitrile after 40% acetonitrile precipitation. The two pellets and the last supernatant were taken together with the original whole plasma for the analyses. (B) Heatmap to demonstrate the distinctly enriched subsets of proteins identified in these four samples. (C) Venn diagram to show unique and shared proteins identified in these samples. (D–F) Analyses of differences in the protein sizes, hydrophobicity, and the electric points of the identified proteins among these four samples. MW: molecular weight. The Gravy score is the indicator of the hydrophobicity of a protein. (G) Significantly enriched domains in proteins of these four samples.

were reduced to about 25% while it was around 70% as usual in the original whole plasma (Figure 1B). With more proteins starting to be precipitated, the percentage of albumin also increased. At 50% acetonitrile, albumin in the precipitated pellet increased to ~80% (Figure 1B). We also performed SDS-PAGE and Coomassie blue staining to visualize proteins in these precipitates and the final supernatant and found that the intensities of protein bands above albumin were generally stronger at 10% and 20% acetonitrile than those in the original

plasma and became weakened at 30% and 40% acetonitrile (Figure 1C). At 50% acetonitrile, albumin became the main protein in the pellet. These data suggested that acetonitrile preferentially precipitated many proteins other than albumin at certain concentrations.

We next performed proteomics analyses on these samples. Each sample showed different liquid chromatographs, indicating distinct protein contents of these samples (Figure 1D). This was also demonstrated in the heatmap analysis of the proteomic

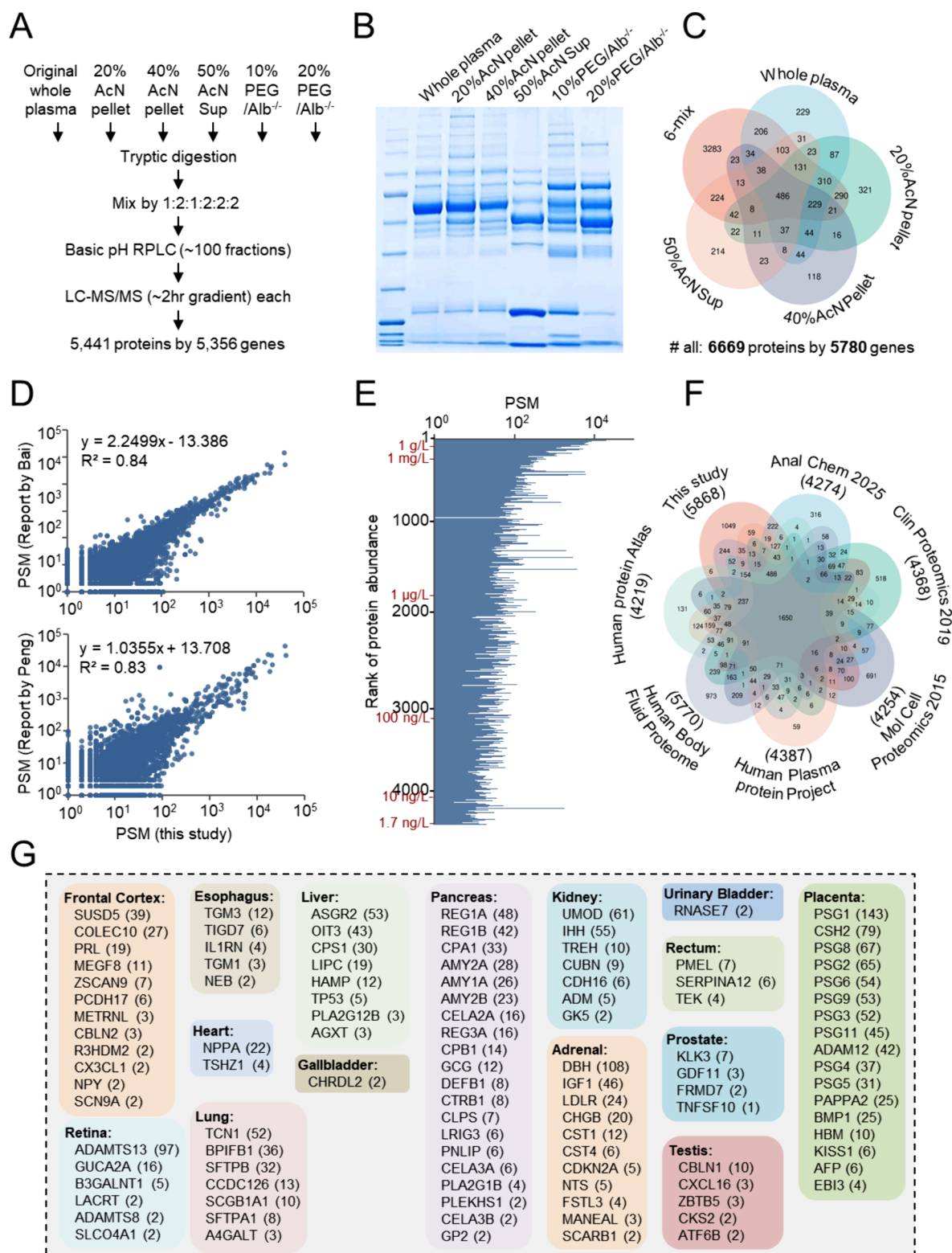


Figure 3. In-depth proteomic analysis of the mixture of the six samples. (A) Work flow of the in-depth proteomic analyses. The plasma was precipitated by 20%, 40%, or 50% acetonitrile, and the pellets and the final supernatant were collected. The plasma was also precipitated by 10% or 20% polyethylene glycol 6000, and then the supernatants were both filtered to deplete albumins before collection. The original whole plasma and its five isolates were mixed at the indicated ratios for the in-deep proteomic analysis. (B) SDS-PAGE and Coomassie blue staining of the plasma and its five isolated fractions. (C) Venn diagram to show unique and shared proteins identified in the in-depth analyses of the six mixed samples and the previous four samples. (D) Correlation of the proteins identified in this 6mix analysis with the two reported in-depth plasma proteomic analyses.^{9,20} (E) Proteins sorted by their absolute concentrations [from the highest (40 g/L of the albumin) to the lowest (1.7 ng/L of the Interferon lambda-3)] provided in the Human Protein Atlas database. (F) Comparison of proteins identified in the 6mix of this study with other reported (PMID: 40468192, 31019427, 25724909) or recorded plasma proteins in databases. (G) Tissue-specific proteins identified in this in-depth proteomic analysis of the plasma 6mix sample.

results of these samples (Figure 1E). In the total 391, 434, 339, 323, 308, 275, and 193 proteins identified in the six pellets and the final supernatant (Supplemental Figure S6), there were 79, 90, 20, 34, 39, 86, and 55 proteins uniquely identified in all these seven samples (Figure 1F). It is notable that although 10% and 20% acetonitrile had precipitated a smaller mass of proteins, total proteins and unique proteins were both much more than any other samples.

In-Depth Proteomics Analyses of the Acetonitrile-Precipitated Pellets and Supernatant

To look into proteins in these samples more comprehensively, we chose the pellet of plasma precipitated by 40% acetonitrile directly (40P) as it seemed to contain similar proteins to the original plasma but less amount of albumin (Figure 1C), the pellet of plasma precipitated by 50% acetonitrile (50P) after 40% acetonitrile precipitation which contained largely albumin, the final supernatant (50Sup) of the 50% acetonitrile supernatant, together with the original plasma (Plasma) (Figure 2A, Supplemental Figure S7A–D). By extensive basic pH reverse phase fractionation of peptides and analyzing individually without concatenation, 2040, 2078, 1153, and 1414 proteins were identified in the Plasma, 40P, 50P and 50Sup samples, respectively, yielding 3386 proteins identified in total (Figure 2A), in which the result of the 50Sup seemed to extend the proteome depth more markedly (Supplemental Figure S7E). The heatmap analysis demonstrated that a large number of proteins were enriched in the Plasma, 40P and 50Sup while fewer were present in 50P (Figure 2B), consistent with the results that 50P mainly contained albumin (Figure 1C). Particularly, there were 435, 611, 141, and 438 proteins uniquely identified in these four samples (Figure 2C). Some biological processes, cellular components, and molecular functions were enriched in each of these four samples, but the numbers of hits were all small (Supplemental Figure S8). This suggests that this acetonitrile precipitation strategy is not suitable for analyzing proteins in plasma at the system level from any particular biological processes, cellular components, and molecular functions, meaning it is only applicable for detecting specific proteins of interest.

We also analyzed potential factors that contribute to the differential protein-precipitating effects of acetonitrile by looking into the protein length, hydrophobicity, and isoelectric points across these samples. All three of these factors showed significant differences among the four samples by ANOVA analyses (Figure 2D–F). The average sizes of proteins in the 50Sup sample were lower than those of the other three samples (Figure 2D), consistent with the SDS-PAGE results (Figure 1C). The GRAVY scores, indicators of protein hydrophobicity, were statistically different among these four samples, but the difference was small (Figure 2E). The isoelectric points in the 50Sup sample were also lower than those of the other three samples possibly because a large number of proteins were precipitated (Figure 2F), resulting in a bias in distribution of isoelectric points of the proteins left in the final supernatant.

We next examined whether particular domains might have a significant effect on the protein precipitation by acetonitrile. Although more than several protein domains were enriched in each of these four samples, the number of proteins that contain these was only up to 32 which is negligible as compared to more than 1000 proteins in these samples (Figure 2G). Collectively, the protein sizes, hydrophobicities, isoelectric points, and

particular domains might have small effects on the sequential protein-precipitating effects of acetonitrile.

Mixture of Plasma and Its Isolates for In-Depth Analysis

As pellets and supernatant after acetonitrile precipitation were able to enrich subsets of proteins, combining them at certain ratios might be able to increase the number of proteins to be detected. We also considered combining the fractions from plasma after PEG precipitation and albumin depletion as we recently developed.²⁰ Therefore, we combined the plasma with the five isolated fractions (6mix) that included a pellet of 20% acetonitrile precipitation, a pellet of 40% acetonitrile precipitation, a supernatant of 50% acetonitrile precipitation, and albumin-depleted supernatants of 10% and 20% PEG6000 precipitation at the ratio of 1:2:1:2:2:2 (Figure 3A, Supplemental Figure S9A). In this pooling recipe, as 40% acetonitrile has largely precipitated proteins from plasma (Figure 1B,C) and thus has similar protein contents to plasma (Figure 3B), they were equally mixed as one sample to be pooled with the other four extracts equally.

The SDS-PAGE and the liquid chromatographs demonstrated distinct patterns among these six samples, indicating their different proteomes that could be complementary to one another (Figure 3B, Supplemental Figure S9B). By extensive high pH reverse phase fractionation and analyzing individually without concatenation, 5441 proteins encoded by 5336 genes were finally identified (Figure 3A, Supplemental Figure S9A,C), including 3987 reviewed proteins, 4381 proteins with PSM ≥ 2 , 4853 proteins with FDR < 0.01 (Supplemental Figure S9D) among which there were 3237 proteins in reviewed status with at least 2 PMS at FDR below 0.01. Proteins identified in the 6mix sample were largely enriched (Supplemental Figure S10), and there were 3283 proteins uniquely present in the 6mix sample (Figure 3C).

Comparing to our previous deep plasma proteomics reports, which used similar proteomics analysis pipelines, this study showed high correlation with both reported analyses (Figure 3D). To estimate how deep this mixture strategy could find proteins in the plasma, we downloaded the absolute concentrations of proteins from the human protein atlas database and ranked them from high to low levels and found that proteins even ranked at the bottom where the levels ranging from a few to tens of ng/L could have more than 100 PSMs (peptide-spectrum matches, the number of a protein or a peptide sequenced) (Figure 3E). This strongly suggested that proteins at the level of pg/mL could be detected in high chance. Indeed, many protein markers routinely assayed in clinic such as CEA (carcinoembryonic antigen), AFP (alpha-fetoprotein), PSA (prostate-specific antigen), NSE (neuron-specific enolase), etc., were detected in this study (Supplemental Figure S5, S11).

Comparing the plasma proteomics studies from the leading groups and the public plasma protein databases, we found 1049 proteins that were not reported or recorded before (Figure 3F).

Since a large number of proteins were identified in this study and the plasma sample was largely from clinical patients with different diseases, we checked to see if there were any tissue-specific proteins that could be used as disease biomarkers. Based on the human tissue proteome analysis, many tissue specific or highly enriched proteins were actually detectable in the plasma of this study and could potentially be blood biomarkers indicating lesions of these tissues (Figure 3G).

Table 1. Novel Plasma Proteins Identified in the 6mix Sample^a

Accession	Gene	Protein	Exp. q-value	Confidence	PSM	# of all peptides	# of unique peptides	Involvement in diseases	Reference (PMID)
Q9Y2L1	DIS3	Exosome complex exonuclease RRP44	0.00	High	22	13	13	Colorectal cancer, chronic lymphocytic leukemia and multiple myeloma	26193331
P49207	RPL34	Large ribosomal subunit protein eL34	0.00	High	22	6	6	Osteosarcoma and Diamond-Blackfan anemia	27883047
Q96E39	RBMXL1	RNA binding motif protein, X-linked-like-1	0.00	High	21	7	1	Overexpressed in individuals with acute myeloid leukemia	34458856
Q9BTE3	MCMBP	MCM-binding protein	0.00	High	19	11	11	Highly abundant in colorectal adenocarcinoma	25246271
Q96J01	THOC3	THO complex subunit 3	0.00	High	19	11	11	Highly expressed in glioma cells with poor prognosis	34482648
Q96RP9	GFM1	Elongation factor G, mitochondrial	0.00	High	17	7	7	Neurological disorders with other different complications	31680380
Q7L0Y3	TRMT10C	tRNA methyltransferase 10 homologue C	0.00	High	17	11	11	Early death after birth, lung cancer and hepatoblastoma	27132592
Q9BQ04	RBM4B	RNA-binding protein 4B	0.00	High	16	9	9	Might be mutated amyotrophic lateral sclerosis	29170628
Q96DI7	SNRNP40	U5 small nuclear ribonucleoprotein 40 kDa protein	0.00	High	15	7	7	Mutated in immune disorder and cancer	31427773
Q01081	U2AF1	Splicing factor U2AF 35 kDa subunit	0.00	High	15	8	8	Mutated in myelodysplastic syndromes and myeloid malignancies	22158538
J3KQN4	RPL36A	Ribosomal protein L36a	0.00	High	14	7	7	Oral squamous cell carcinoma, colorectal and hepatocellular cancers	34830778
A0A087WW65	ABCB7	Iron-sulfur clusters transporter ABCB7, mitochondrial	0.00	High	13	7	7	Involved in acquired refractory anemia with ring sideroblasts	23070040
P51116	FXR2	RNA-binding protein FXR2	0.00	High	13	7	4	Fragile X mental retardation syndrome and breast cancer	7489725
Q15031	LARS2	Leucine-tRNA ligase, mitochondrial	0.00	High	13	9	9	Mutated in Perrault syndrome	32423379
P53985	SLC16A1	Monocarboxylate transporter 1	0.00	High	13	4	4	Causes recurrent ketoacidosis and involved in urological cancer	26608392
Q8IU68	TMC8	Transmembrane channel-like protein 8	0.01	High	13	1	1	Cancers and epidermodysplasia verruciformis	33981360
C9JG87	MRPL39	Mitochondrial ribosomal protein L39	0.00	High	12	8	8	Pediatric onset mitochondrial disease	37133451
P62273	RPS29	Small ribosomal subunit protein uS14	0.00	High	11	2	2	Diamond-Blackfan anemia	24829207
Q5T8P6	RBM26	RNA-binding protein 26	0.00	High	11	8	8	(Little direct evidence in clinical diseases)	
Q9H078	CLPB	Mitochondrial disaggregase	0.00	High	11	8	2	(Little direct evidence in clinical diseases)	
P14678	SNRPB	Sm protein B/B'	0.00	High	11	6	6	3-Methylglutaconic aciduria and neurological disorders	25597510
B8ZZY4	SLC35F5	Solute carrier family 35 member F5	0.00	High	10	1	1	Might be involved in bladder cancer	33418944
Q3SXM5	HSDL1	Inactive hydroxysteroid dehydrogenase-like protein 1	0.00	High	10	3	3	(Little direct evidence in clinical diseases)	
P11802	CDK4	Cyclin-dependent kinase 4	0.00	High	10	6	5	Many cancers and causes insulin-deficient diabetes	35304604
P62891	RPL39	Large ribosomal subunit protein eL39	0.00	High	10	2	2	Mutated in cancer and promotes tumorigenesis	28040796
Q04727	TLE4	Transducin-like enhancer protein 4	0.00	High	10	7	4	Involved in acute lymphoblastic leukemia	39838044
Q96G23	CERS2	Ceramide synthase 2	0.00	High	9	2	2	Steatohepatitis, insulin resistance and cancer	25295789
Q86Y56	DNAAF5	Dynein axonemal assembly factor 5	0.00	High	9	6	6	Might be involved in hepatocellular carcinoma	36276075
Q5SRKV6	EXOSC6	Exosome complex component MTR3	0.00	High	9	3	3	(Little direct evidence in clinical diseases)	
B4DT23	FAM98A	Family with sequence similarity 98 member A	0.00	High	9	3	1	Involved in cancer	31114934
E7ESY4	MTA1	Metastasis associated 1	0.00	High	9	2	1	Involved in cancer	25344802
G3 V599	MIA2	MIA SH3 domain ER export factor 2	0.00	High	8	7	7	A tumor suppressor in hepatocellular carcinoma	17881540
A0A8Q3SHU8	MRPS25	Small ribosomal subunit protein mS25	0.00	High	8	5	5	Mutations cause encephalomyopathy	31039582
G5EA06	MRPS27	Mitochondrial ribosomal protein S27	0.00	High	8	8	8	Highly expressed in cancer, involved in ischemic stroke	37603533

Table 1. continued

Accession	Gene	Protein	Exp. q-value	Confidence	PSM	# of all peptides	# of unique peptides	Involvement in diseases	Reference (PMID)
Q9HCU5	PREB	Guanine nucleotide-exchange factor SEC12	0.00	High	8	5	5	Regulates hepatic glucose homeostasis	29601978
P18577	RHCE	Blood group Rh(CE) polypeptide	0.00	High	8	2	2	Blood group antigen	
A8MQ02	AFDN	Afadin, adherens junction formation factor	0.00	High	7	6	6	Involved in metastatic colorectal cancer	39047222
A0A8I5KRG1	DCAF7	DDB1 and CUL4 associated factor 7	0.00	High	7	4	4	(Little evidence in clinical diseases)	
Q8WVC6	DCAKD	Dephospho-CoA kinase domain-containing protein	0.00	High	7	5	5	Genetic variations in cerebral small vessel disease	34987231
P29992	GNA11	G-protein subunit alpha-11	0.00	High	7	5	1	Involved in uveal melanoma	35804836
Q9BXW7	HDHD5	Cat eye syndrome critical region protein 5	0.00	High	7	7	7	(Little direct evidence in clinical diseases)	
C9JBY7	MRPS33	Small ribosomal subunit protein mS33	0.00	High	7	3	3	(Little direct evidence in clinical diseases)	
C9JYQ9	RPL22L1	Ribosomal protein L22 like 1	0.00	High	7	3	2	Involved in many cancers	40022129
A0A0G2JQ92	TAMM41	Phosphatidate cytidylyltransferase, mitochondrial	0.00	High	7	1	1	Neonatal mitochondrial diseases and depressive-like behaviors	38750395
Q9BW92	TARS2	Threonine-tRNA ligase, mitochondrial	0.00	High	7	6	6	Biallelic variants cause neurodevelopmental aberrancy	37454282
C9JG97	AAMP	Angio associated migratory cell protein	0.00	High	6	5	5	Promotes colorectal cancer metastasis	34901393
Q6IA86	ELP2	Elongator complex protein 2	0.00	High	6	2	2	Mutations cause complex neurodevelopmental aberrancies	33976153
H7C5U8	MRPL27	Mitochondrial ribosomal protein L27	0.00	High	6	3	3	Cholangiocarcinoma as a poor prognostic marker	33456351
H0Y6Y8	MRPL43	Large ribosomal subunit protein mL43	0.00	High	6	4	4	Genetic association with dementia with Lewy bodies	35765761
A8MXV4	NUDT19	Acyl-coenzyme A diphosphatase NUDT19	0.00	High	6	5	5	(Little direct evidence in clinical diseases)	
P24928	POLR2A	DNA-directed RNA polymerase II subunit RPB1	0.00	High	6	5	5	Neurodevelopmental aberrancy and cancers	31353023
A0A087WXB0	SCAMP1	Secretory carrier-associated membrane protein	0.00	High	6	3	3	Involved in gastric cancer	39308691
Q13190	STX5	Syntaxin-5	0.00	High	6	3	3	Involved in Hepatocellular Carcinoma	36969886
C9JHH5	TEX264	Testis expressed 264, ER-phagy receptor	0.00	High	6	5	5	Regulator in nutrient stress	31006537
Q6NZY4	ZCCHC8	Zinc finger CCHC domain-containing protein 8	0.00	High	6	3	3	Mutations in pulmonary fibrosis	38375433

^aProteins with high confidence (FDR < 0.01), PSMs of 6 or more and with unique peptides were listed.

Novel Plasma Proteins Identified in This Study

There were 6,669 proteins identified of the plasma in this study for the five in-depth proteome data sets (Figure 3C) which is probably the largest coverage in a single plasma sample. We therefore checked to see whether there were any novel plasma proteins that have not been reported in studies or recorded in public databases. We chose four large-scale plasma proteomics studies,^{7,9,20,33} three plasma protein databases (Human Plasma Proteome Project Data,⁴ Human Body Fluid Proteome database,³⁰ Human protein Atlas³⁴) and the SomaScan 7k (7596 probes)³⁵ to combine them for this study for comparison. There were 672 proteins identified in this study that were not present in these reports and databases (Supplemental Table S2). Among these there were 55 proteins with PSMs of 6 or more, FDR < 0.01, and at least one unique peptide identified in the repartitioned sample (6mix), many of which are involved in important clinical diseases (Table 1) and could thus be potential biomarkers of these diseases. The PSMs of these novel proteins are generally around 10. According to the absolute plasma protein concentrations provided by the Human Protein Atlas database, in which identified proteins at the range of the lowest concentration (1–10 ng/L) are around 10 PSMs (Figure 3E),

the novel proteins identified in this study are therefore estimated to be as close to 1 ng/L.

DISCUSSION

In this study, we used acetonitrile to sequentially precipitate proteins from the plasma and chose some of the pellets and supernatant in combination with the fractions of plasma isolated by our other reported PEG-precipitation/albumin-depletion method²⁰ for in-depth proteomics analysis. This plasma sample protein-repartitioning strategy has surprisingly led to profiling of 5,441 proteins in the plasma, reaching probably the largest scales in the plasma proteomics field.

Generally, the number of proteins identified in MS-based plasma proteomics is about a few thousands.^{14,16,18,33,36} In studies with leading scales of plasma proteomics, Dr. Carr's group was able to identify 4,600 proteins in average from 30 fractions of the plasma samples with depletion and 3 h of gradient in each run,⁷ recommended as a standard plasma proteomics pipeline.⁶ By 180 fractions and 3 h running time, Dr. Peng's group profiled 4,826 proteins from the undepleted human serum samples.^{8,9} Using depletion and simple fractionation techniques, Dr. Poljak and colleagues profiled around 4,000

proteins from 24 concatenated peptide fractions by 1-h run per fraction sample.¹⁰

In this study, by our plasma protein repartitioning strategy, without depletion of commonly abundant proteins, we were able to identify more than 5,000 proteins from 2 h gradient runs of about 100 unconcatenated fractions, representing almost the largest scale of the MS-based plasma proteomics studies.

The major reason for this remarkably improved proteome coverage is protein repartition during plasma processing. For example, although the yield of 20% acetonitrile precipitation is low (only about 120 μ g from 100 μ L plasma), some proteins are already largely precipitated (Supplemental Table S1). Compared to plasma in which the total protein is about 7,000 μ g per 100 μ L, these proteins will be enriched by nearly 60-fold (Figure 1B,C). Besides, the percentage of albumin content in the total precipitated proteins is much lower than in plasma, conferring less interference on detection of other proteins. This also accounts for the fact that the numbers of all proteins and unique proteins identified in these precipitates are the largest among all of the sequential acetonitrile precipitates (Figure 1F). The 40% acetonitrile precipitate has the majority of the proteins of the original plasma but contains lower relative level of albumin (Figure 1B,C). Besides, by PEG precipitation and albumin depletion, commonly abundant proteins are largely removed and other proteins are thus enriched for increased chance of being detected.²⁰ By this plasma protein repartition strategy, proteins with levels that are low down to ng/L are detectable (Figure 3E).

The other factor that contributes to the increased number of identified proteins comes from the pooling of plasma from individuals with many different types of diseases, in which many proteins were upregulated. It is notable that with more sophisticated mass spectrometer and scanning methodologies (such as the data-independent acquisition mode),^{37–39} the number of identified proteins could probably increase much more. The extensive DDA data set in this study could serve as a foundation for generating a custom DIA spectral library to support DIA-based analyses in the plasma proteomics research field.

Here, by plasma sample protein repartitioning, we were able to identify more than 5,000 proteins from the plasma, reaching probably the largest scale in the MS-based plasma proteomics field. This plasma sample processing method and extensive peptide fractionation could be easily adapted to the TMT-based in-depth quantitative proteomic analysis of clinical samples at large scales for increased success in blood proteomic biomarker discoveries.

■ ASSOCIATED CONTENT

Data Availability Statement

All MS raw data and search results have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository^{40,41} with the data set identifier PXD065609.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.5c00814>.

Figure S1. Plasma precipitated by different concentrations of acetonitrile; Figure S2. Changes in the levels of routine biochemical analytes in plasma after precipitation by different concentrations of acetonitrile; Figure S3. Clinical information on patients or the hospital visitors

from whom the 100 plasma samples were collected for pooling; Figure S4. Levels of the routine clinical biochemical analytes of the pooled plasma samples; Figure S5. Levels of the common clinical protein markers of the pooled plasma samples; Figure S6. Identified proteins in seven AcN precipitation samples; Figure S7. Precipitation of the plasma sample and proteomic analyses; Figure S8. Gene ontology analyses of the plasma and its three isolated fractions; Figure S9. Plasma isolation and mixture for in-depth proteomic analyses; Figure S10. Heatmap to demonstrate the in-depth proteomic analyses of the plasma 6mix sample and the original whole plasma with its three isolated pellets or the supernatant; Figure S11. Heatmap to demonstrate the in-depth proteomic analyses of the plasma 6mix sample and the original whole plasma with its three isolated pellets or the supernatant (PDF)

In-depth proteomics data sets (XLSX)

Novel identified plasma proteins (XLSX)

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Funding

This work was supported by the National Natural Science Foundation of China (82172354, 82472333, to B.B.), the Research Foundation of Jiangsu Provincial Commission of Health and Family Planning (M2021012, to B.B.), the Nanjing Medical Science and Technology Development Foundation (ZKX22013, to B.B.), the fundings for Novel technology and Clinical Trials from the Affiliated Drum Tower Hospital, Medical School of Nanjing University (XJSFZLX202331 and 2022-LCYJ-MS-02, to B.B.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Nanjing Jiangbei New District Biopharmaceutical Public Service Platform (Nanjing, China) for technical support and all the lab members for helpful discussion.

ABBREVIATIONS

AcN, acetonitrile; PEG, polyethylene glycol; FDR, false discovery rate; LC, liquid chromatography; MS, mass spectrometry; ANOVA, analysis of variance.

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