SUPPORTING INFORMATION

Assessment of urine sample collection and processing variables for extracellular vesicle-based proteomics

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EXPERIMENT SECTION

1. Materials and reagents

EVtrap beads are provided by Tymora Analytical Operations (West Lafayette, Indiana). Triethylamine, sodium deoxycholate (SDC), sodium lauroyl sarcosinate(SLS) , 2-Chloroacetamide(CAA), triethylammonium bicarbonate buffer (TEAB), ethyl acetate(EA) ,trypsin, formic acid(FA), tris(2-carboxyethyl)phosphine(TCEP) were purchased from sigma. Endopeptidase Lys-C (Wako Chemicals), acetonitrile (ACN, Fisher Scientific), CD9 primary antibody (13403S, CST), CD81 primary antibody (ab79559, Abcam), TSG101 primary antibody (ab125011, Abcam), Calnexin primary antibody (ab133615, Abcam), polyvinylidene difluoride (PVDF) membrane (Millipore). Bovine serum albumin (BSA), Glycine, Sodium dodecyl sulfate (SDS), 30% acrylamide-bisacrylamide, Tris Buffered Saline with Tween20 (TBST) were purchased from Biosharp. Other reagents are common laboratory reagents without specific supplier requirements.

2. Western Blotting Analysis

The extracted EVs were detected by WB with four different antibodies, and the cell extract was used as the control. The obtained vesicles were dissolved in SDS-PAGE sample buffer and incubated at 95 ℃ for 10 minutes, and then separated on SDS-PAGE gel. The protein was transferred to PVDF membrane. After the membrane was transferred, the membrane was sealed with TBST solution containing 1%BSA, and then the membrane was incubated with anti-CD9, anti-CD81, anti-TSG101 and anti-Calnexin primary antibodies with the proportion of 1:2000 overnight. After overnight, the membrane was specifically bound to the second antibody coupled with HRP in 1%BSA in TBST. Finally, the target band was visualized by Tanon5200 image analyzer.

3. LC−MS Sample Preparation

First, the isolated EVs were dissolved in a solution containing a mixture of 12 mM sodium deoxycholate, 12 mM SLS, 10 mM Tris-HCl, and 40 mM CAA (pH 8.5). Then, the mixture was boiled for 10 minutes at 95°C in a water bath, followed by the addition of four times the volume of 50 mM triethylammonium bicarbonate. Lys-C enzyme was added at an enzyme-to-protein ratio of 1:100 (w/w), and the mixture was digested at 37°C for 3 hours. After 3 hours, trypsin was added at an enzyme-to-protein ratio of 1:50 (w/w), and the mixture was incubated overnight at 37°C. Following the overnight incubation, the samples were acidified to a final concentration of 1% TFA by adding 10% trifluoroacetic acid (TFA), and an equal volume of ethyl acetate was added to the mixture. The resulting solution was vortexed for 2 minutes and centrifuged at 17,000 × g for 3 minutes. The upper organic phase was discarded, and the lower aqueous phase was collected and lyophilized using a freezing vacuum centrifuge (Labconco CentriVap). The desalting process was performed using a desalting membrane (3M Empore 2240-SDB-XC) according to the manufacturer's instructions, and the desalted solution was lyophilized and ready for subsequent mass spectrometric detection.

SUPPLYMENTARY FIGURES

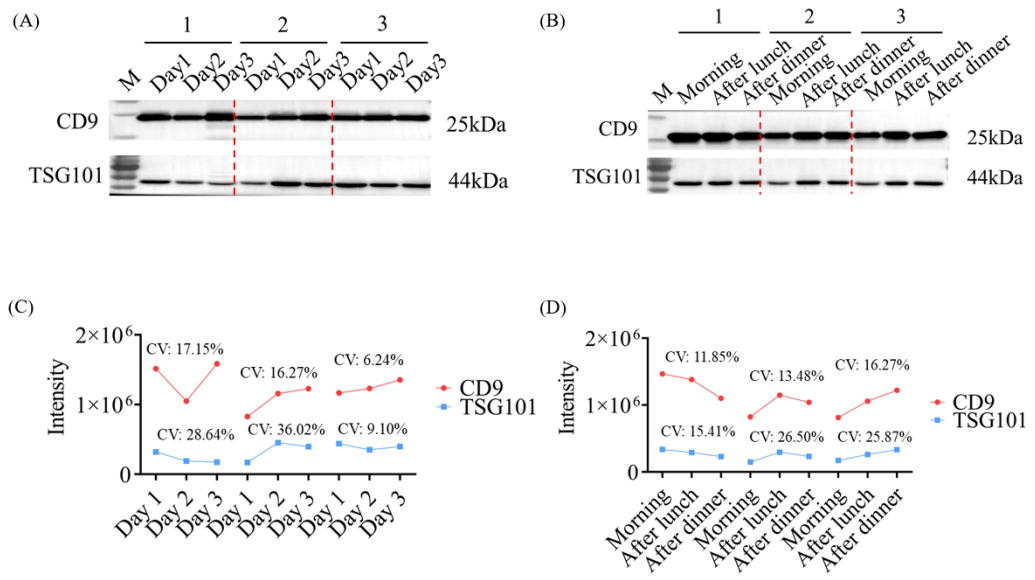

Figure S1. Characterization of EVs in urine. (A) The WB results of EVs in morning urine were observed 3 consecutive days(n=3), (B) The WB results of EVs in morning urine were observed within 1 days (n=3), (C) The coefficient of variation of EVs in morning urine was calculated for 3 consecutive days (n=3), (D) The changes of EVs in urine throughout the day, including morning, after lunch, and after dinner (n=3).

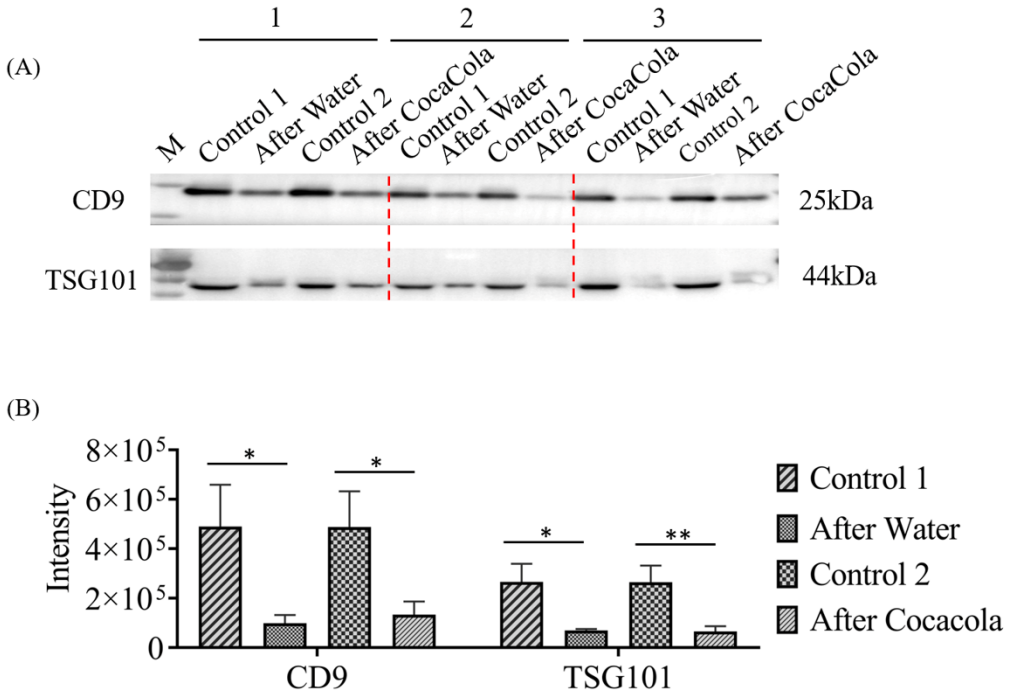

Figure S2. Characterization of EVs in urine under extreme dietary conditions. (A) Results of WB detection of EVs in urine under extreme dietary conditions (n=3), (B) Grayscale value of CD9 and TSG101 bands in WB result (n=3). *p ≤ 0.05, **p < 0.01, ***p < 0.001.

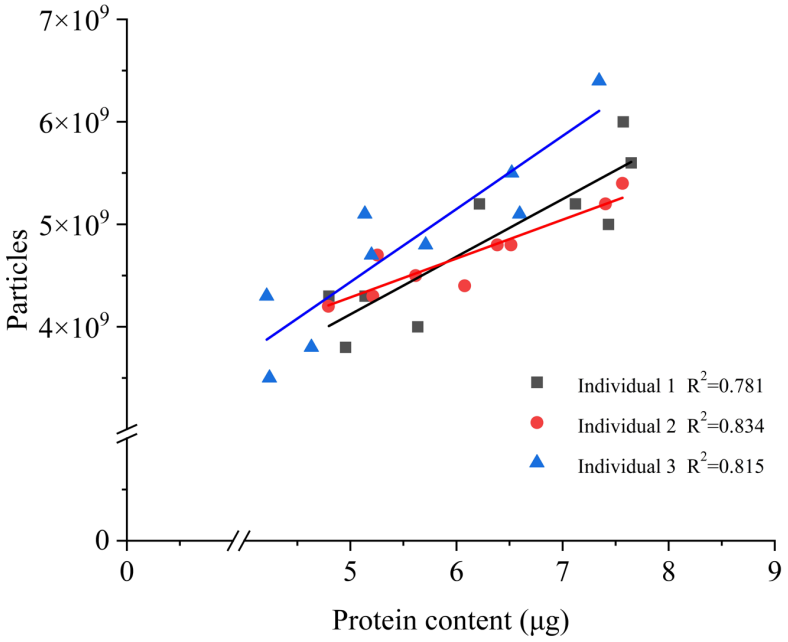

Figure S3. Correlation between urinary EV number and total protein content.

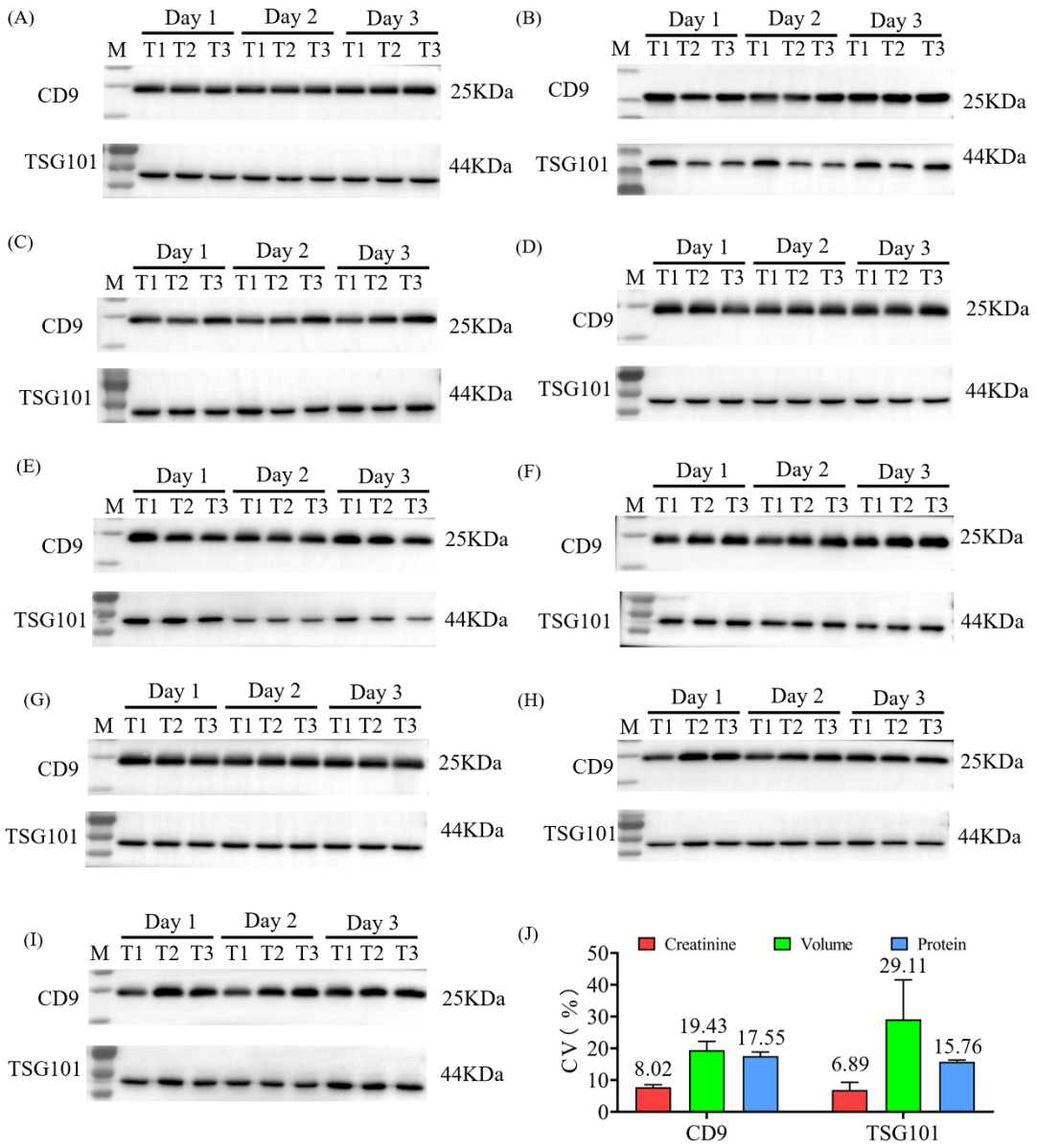

Figure S4. Comparison is made between different normalization methods. (A-C) WB results are shown after applying 3 normalization methods to individual 1 (creatinine, volume, and protein, in order). (D-F) WB results are shown after applying 3 normalization methods to individual 2 (creatinine, volume, and protein, in order). (G-I) WB results are shown after applying 3 normalization methods to individual 3 (creatinine, volume, and protein, in order). (J) A comparison is made between different normalization methods. M: Marker, T1: Time 1, T2: Time 2, T3: Time 3.

(A)

(B)

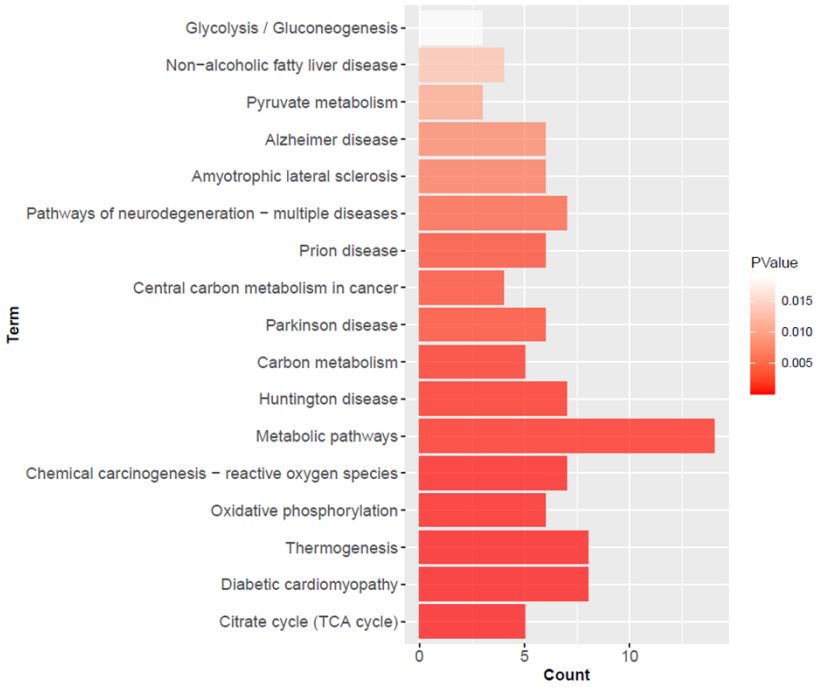

Figure S5. (A) GO analysis of these upregulated proteins. (B) KEGG pathway analysis of these upregulated proteins.

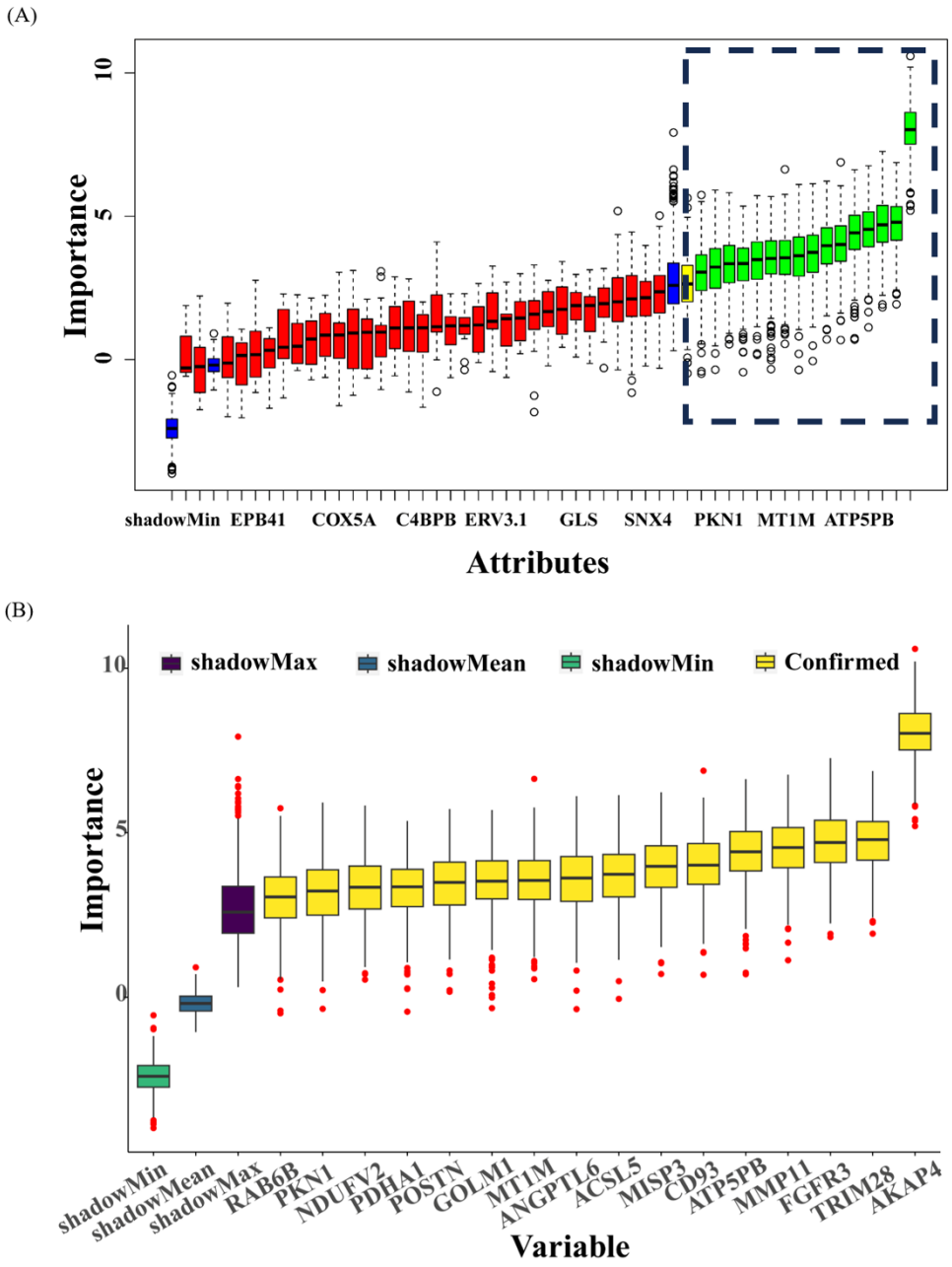

Figure S6. (A) Characterization screening of 51 up-regulated proteins. (B) The variable importance plots (VIPs) for the 16 differential proteins.

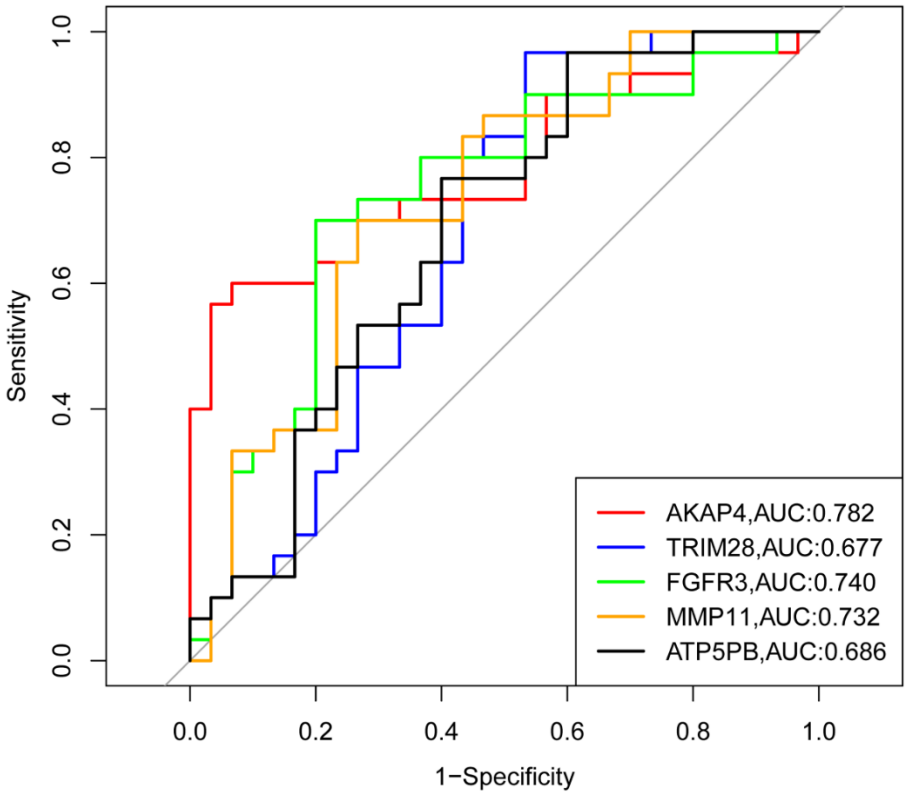

Figure S7. The receiver operating characteristic (ROC) curve for the top 5 proteins obtained from the VIPs.

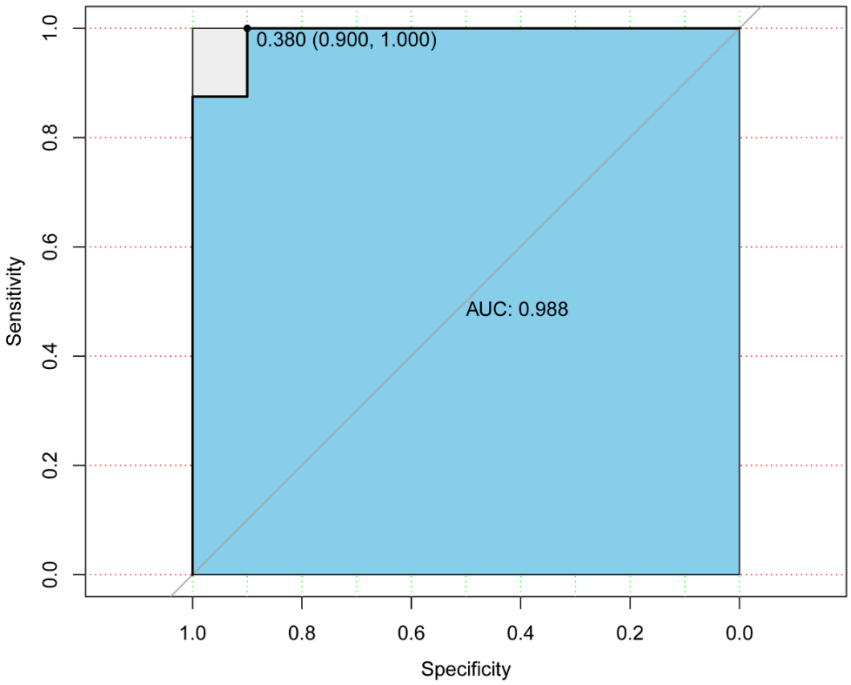

Figure S8. The ROC of the panel for the 5 feature proteins obtained from the VIPs.