RESEARCH ARTICLE

A stable panel comprising 18 urinary proteins in the human healthy population

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Just as biomarkers specific for diseases, biomarkers indicative of healthy conditions are valuable for the early diagnosis, monitoring, and prognosis of diseases. Our study focused on discovering via proteomics a stable panel of urinary proteins in the human healthy population. Urine samples were collected three times during 4 months from 100 male and 100 female healthy donors and analyzed through four different fractionation techniques (i.e. in-gel, 2D-LC, OFFGEL, and mRP) coupled with HPLC-Chip-MS/MS. Thus, 1641 urinary proteins were identified with a high confidence, among which 70 exhibiting an intergender/day variation <0.25 were selected and matched with the previously published five largest urinary proteomes to get 56 candidate proteins. Next, a panel comprising 18 intact urinary proteins was constructed by comparing the urinary proteomes via SDS-PAGE and 2DE. Finally, such 18 urinary proteins were validated via enzyme-linked immunosorbent assay in eight healthy individuals. Most of these proteins had been related to multiple rather than to single diseases. Therefore, we surmise that this protein set could be used as a biomarker to assess the human health status. Further determinations of the normal fluctuations of the single urinary proteins in this series using samples from large numbers of healthy individuals are required prior to any application in clinical settings.

Keywords:
Biomarker / Biomedicine / Dynamic variation / Mass spectrometry / Urinary proteomics

1 Introduction

Human urine is one of the ideal sources for clinical diagnosis, especially for urinary system diseases, as urine can be obtained noninvasively, in large quantities, and repeatedly. Urinary proteins might therefore be an ideal source of biomarkers for several diseases [1]. As most illnesses are complex and heterogeneous processes, it would be preferable to use a constellation of individually informative proteins rather than a single one to assess the diseased condition. By means of comparative proteomics, a lot of urinary proteins have been reported as potential biomarkers for bladder cancer [2], renal cell carcinoma [3], rejection of kidney transplants [4, 5], acute kidney injury [6, 7], Fanconi syndrome [8], renal failure in diabetic nephropathy [9], and others. However, most biomarker candidates are likely to be related to multiple diseases rather than to a single one. It should be reasonable to assume that such proteins reflect human healthy conditions, and that their changes in expression may indicate that the people involved are affected by an unhealthy condition. Therefore, establishing a secure panel of urinary proteins reflecting human healthy conditions would not only provide a

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reference set for identifying disease biomarkers, but also be valuable for the early diagnosis, monitoring, and prognosis of diseases. Our present study is focused on discovering urinary biomarkers indicative of human healthy conditions through proteomic approaches.

Even though many intensive studies on the normal urinary proteome have been performed [10–14], a steady panel of urinary proteins in the healthy human population has not yet been established due to the considerable degree of dynamic variation proper of the urinary proteome [14–17]. It is well known that urinary proteomes differ among individuals, particularly between men and women. In addition to these interindividual differences, the urinary proteome of the same person varies at different times due to the effects of exercise, diet, lifestyle, and other factors [14–17]. Here, to determine a steady panel of urinary proteins in the human healthy population, we first controlled on a large scale the individual dynamic variations by collecting the urine samples from 200 healthy donors aged 18–22 years. Next, the urinary proteins with the dynamic intergender/day variations <0.25 were selected and matched with the previously published five largest urinary proteomes; thus, a reproducible set comprising 18 intact urinary proteins was obtained via comparisons with the urinary proteomes based on SDS-PAGE and 2DE [18, 19]. Finally, the 18 urinary proteins were validated by ELISA in eight healthy individuals. Most proteins in this set were shown to be linked to multiple rather than single diseases. Therefore, it seems reasonable to assume that this protein set would constitute a reference marker revealing the human health status.

2 Materials and methods

2.1 Preparation of urinary proteins from healthy people

The protocol for collecting urine samples was approved by the Ethics Committee of the Southwest Hospital, Chongqing. The samples were collected thrice, at zero time and after 2 and 4 months, from 100 healthy male and 100 healthy female Chinese volunteers with an age range of 18–22 years, who underwent medical checkups and urine routine tests at the Southwest Hospital. Clinical details on the study participants are reported in Supporting Information Table S1. Each time, all 100 male and 100 female urine samples were collected on the same day and 10-mL urine was immediately taken from each sample to build the male and female pools, respectively. The pooled samples were centrifuged at 10 000 $\times$ *g* for 30 min at 4°C to remove any cellular debris. The supernatant was concentrated and desalted via tangential filtration in a Lab-scale TFF system (Millipore, Billerica, MA, USA) using a membrane with a cutoff of 10 kDa, and the volumes were reduced to about 30 mL. The protein amount in the urine concentrates was measured by Coomassie Protein Assay Kit (Pierce, Rockford, IL, USA). The targeted high-abundance proteins (i.e. albumin, IgG, antitrypsin, IgA, transferrin, and haptoglobin) in the concentrated samples were depleted by using an immune-affinity column. The amount of urinary protein after depletion of high-abundance proteins was then assessed as mentioned above. Soon after, the urine concentrates were stored at −80°C.

2.2 1D SDS-PAGE and in-gel digestion

After depletion of the high-abundance proteins, the urinary protein mixture (200 μg) was loaded on a 10% Bis-Tris gel (Novex; Invitrogen, Carlsbad, CA, USA) in accordance to the manufacturer's instructions. The gel lane was then cut into 18 pieces and performed in-gel tryptic digestion. After DTT reduction and iodoacetamide alkylation, the proteins were digested by trypsin (Promega, Madison, WI, USA) at 37°C overnight. Then, we used a solution of 30% ACN, 0.3% TFA, and 100% ACN to extract the tryptic peptides. After organic solvents were removed by using a vacuum centrifuge, the extracts were reconstituted in 0.1% TFA for HPLC-MS/MS analysis.

2.3 Reverse phase HPLC and in-solution digestion

The high-abundance protein depleted urinary protein (200 μg) was dissolved in 6 mol/L urea and 1.0% acetic acid. The urinary protein mixture was separated on an mRP-C18 High-Recovery protein column (Agilent Technologies, Palo Alto, CA, USA) at 80°C with a linear multisegment gradient as previously described by Jun Adachi and coworkers [10], and a total of 22 fractions were collected. Each fraction was dried in a vacuum centrifuge and performed in-solution tryptic digestion as previously described by Foster and coworkers [20]. Finally, the resulting peptide mixtures were desalted using reverse phase C18 StageTips and diluted in 0.1% TFA for HPLC-MS/MS analysis.

2.4 OFFGEL electrophoresis

OFFGEL electrophoresis was performed as previously described by Patric Hörth and coworkers [21]. Briefly, the 3100 OFFGEL Fractionator and the OFFGEL Kit 3–10 (Agilent) were applied following the user protocol. A total of 600 μg of the urinary protein tryptic digest was resuspended with focusing buffer to a final volume of 3.6 mL. A total of 150 μL of this sample was loaded in each of the 24 wells. The sample was focused using the recommended method for OFFGEL peptides 24 wells fractionation with a maximum current of 50 μA and until 50 kVh was reached. Corresponding peptides fractions were pooled and concentrated by vacuum centrifugation prior to HPLC-MS/MS analysis.

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2.5 On-line 2D-LC-ESI-MS/MS analysis

A total of 200 μg high-abundance protein depleted urinary protein mixtures were digested in-solution as described above. After desalted by reverse phase C18 StageTips, the protein digest (10 μL) was injected to a strong cation exchange (SCX) column (300 μm i.d. × 5 cm; Agilent Technologies, Waldbronn, Germany) and eluted with ten salt plug injections (5–500 mM NaCl). Eleven fractions obtained from ten salt plug injections were then introduced into the HPLC-Chip/XCT Ultra Trap. The first four fractions were separated with 2-h long gradients (2% B to 40% B in 110 min and 40% B to 95% B in 10 min; where solvent A is 2% ACN, 0.1% formic acid, and solvent B is 0.1% formic acid, 90% ACN) and the remaining fractions with 1-h long gradient.

2.6 HPLC-Chip/MS analysis

The tryptic digested and desalted protein samples were analyzed with a HPLC-Chip-MS/MS system consisting of a nano pump (G2226 A, Agilent) with four-channel microvacuum degasser (G1379B, Agilent), a microfluidic HPLC-Chip Cube interfaced to a XCT Ultra ion trap mass spectrometer (all Agilent Technologies). Peptides were injected on the enrichment column via an autosampler. The mobile phase consisted of solvents A (water with 0.1% formic acid) and B (90% ACN, 10% water with 0.1% formic acid). The column was eluted with a gradient from 3% B to 45% B in 90 min, followed by a steep gradient to 80% B in 10 min. The total analysis time was 110 min, and the flow rate was fixed at 0.3 μL/min.

Data-dependent MS acquisition was performed on the Agilent LC/MSD Trap XCT with the following MS conditions: 4 L/min, 300°C; skim 1: 30 V; capillary exit: 75 V; capillary voltage: 1800 V; for each precursor ion two averages were taken; ion current control: on; trap drive: 85; smart target: 500 000; MS scan range: 400–1600; maximum accumulation time: 150 ms; ultra scan: on. averages: 1; fragmentation amplitude: 1.25 V; MS/MS: number of parents: 5; SmartFrag: on, 30–200%; spectra were actively excluded for fragmentation after two recorded spectra for 1 min to allow the detection of less abundant coeluting compounds; exclude +1: on, MS/MS scan range: 100–2000; prefer +2: on; ion current control target: 500 000; ultra scan: on.

2.7 Database search

Database searches were performed against the IPI human database (http://www.ebi.ac.uk/IPI) with the Spectrum Mill Proteomics Workbench Rev A.03.03.078 software (Agilent Technologies). The Spectrum Mill Data Extractor program was used to create peak lists under the following conditions: scans with the same precursor ±1.4 m/z were merged within a time frame of ±15 s. Charges up to a maximum of five were assigned to the precursor ion. Precursor ions needed with a minimum signal-to-noise value of 25. The ^{12}C peak was determined by the Data Extractor. Two missed cleavages were allowed. Peptides were automatically identified by the Spectrum Mill software using IPI human database (version 3.43) for tryptic peptides with the restriction to *Homo sapiens*. A mass tolerance of ±2.5 Da for the precursor ions and a tolerance of ±0.7 Da for the fragment ions were used. A Spectrum Mill autovalidation was run in protein details mode and the peptide mode. Minimum scores, forward minus reversed score threshold, minimum scored peak intensity, and rank 1 minus rank 2 score threshold for peptides were dependent on the assigned precursor charge (Supporting Information Table S2).

2.8 Enrichment analysis of Gene Ontology (GO) categories

The BiNGO plugin was used to perform an enrichment analysis of our urinary proteome dataset. For enrichment analysis, our identified urinary proteome was used as a test dataset and GO annotation for the complete human proteome as a reference set. The analysis was performed with the "hyper geometric test." After correcting for multiple term testing by Benjamini and Hochberg false discovery rate corrections, the GO terms with $p < 0.001$ were selected as overrepresented or underrepresented. Then, we used ClueGO, an easy way to use the Cytoscape plug-in, to compare our results with Max-Planck Unified (MAPU) urinary proteome GO terms.

2.9 Validation by ELISA

The urine samples were collected for validation from eight healthy donors at five time points within 2 months. The clinical details of these eight individuals are shown in Supporting Information Table S4. After centrifuging the urine samples at 10 000 × g for 30 min at 4°C to remove cellular debris, the supernatant was concentrated and desalted by using a membrane with a cutoff of 10 kDa. The protein amount in the urine concentrates was measured using a Coomassie Protein Assay Kit (Pierce). COL6A1, GC, RBP4, DNASE1, GAA, SERPING1, CTSD, KNG1, UMOD, PIGR, AHSG, A1BG, AMBP, AZGP1, PTGDS, HPX, CD14, and ORM1 proteins were quantified with ELISA kits from Life Science Inc. according the manufacturer's instructions.

2.10 Statistical analysis

The total number of identified MS/MS spectra for any protein was used as the measurement of protein abundance in each

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sample. Protein abundance variation was defined as the ratio of spectrum number standard variation and mean value of a given protein among different samples [17]. Percentages of variances were calculated from the median coefficient of variation (CV), which is the standard deviation divided by the mean of a measurement.

3 Results

The experimental strategy is outlined in Fig. 1. Briefly, the urine samples from 100 male and 100 female healthy donors were pooled together and next concentrated using a 10 kDa cut-off ultrafiltration membrane. The targeted high-abundance proteins in the concentrated urine samples were depleted by means of an immune-affinity column. Next, the processed urinary protein samples were analyzed via four different separation methods (SDS-PAGE (Supporting Information Fig. S1A), mRP (Supporting Information Fig. S1B), OFFGEL, and 2D-LC) coupled with the HPLC-Chip-MS/MS. Tandem mass spectroscopic data were analyzed using the Spectrum Mill software and the IPI database restricted to humans using autovalidation criteria as described above (Supporting Information Table S2).

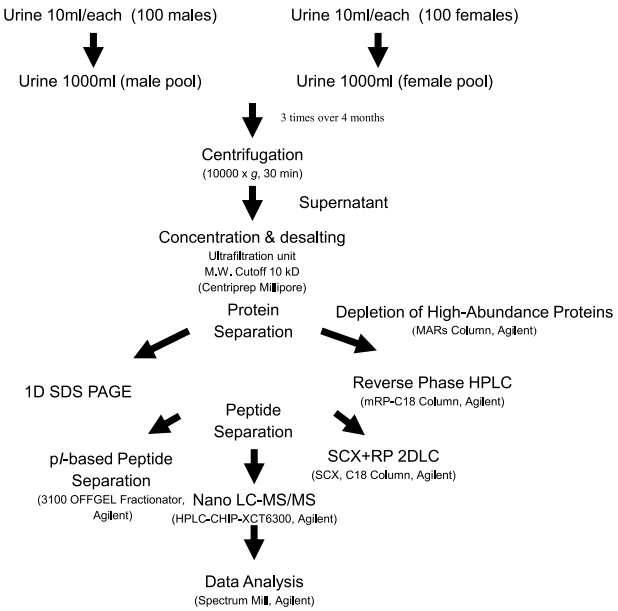

Figure 1. An overview of the procedures used to analyze the human urinary proteomes. HPLC, high-performance liquid chromatography; p*I*, isoelectric point; MW, molecular weight; LC, liquid chromatography; RP, reversed phase; MS, mass spectrometry; 1-D SDS, one-dimensional sodium dodecyl sulfate; MARs, Multiple Affinity Removal System; SCX, strong cation exchange. See the text for details.

3.1 Whole urinary proteome of a human healthy population

3.1.1 Identification of the urine proteome of a human healthy population

A total of 103 901 spectra (5.7%) were ultimately selected and interpreted as validated MS/MS spectra among the total spectra (1 832 582) produced from 456 raw files by the mass spectrometer. Of the 103 901 validated MS/MS spectra, 23 201, 12 733, 44 732, and 23 235 MS/MS spectra were derived from the datasets of SDS-PAGE, 2D-LC, OFFGEL, and mRP, respectively (Supporting Information Table S3). In total, we identified 13 191 unique peptides, which mapped to 1641 nonredundant proteins (Supporting Information Data S1), in which 1212 (73.9%) proteins were identified by at least two unique peptides. Among all the identified peptides, 4485, 2537, 6065, and 3469 unique peptides, which represent 688, 540, 855, and 580 proteins, were derived from the datasets of SDS-PAGE, 2D-LC, OFFGEL, and mRP (Supporting Information Data S1), respectively (Table 1).

3.1.2 Summary of female and male urinary proteomes

We compared the overall features of the urinary proteins between male and female datasets (Supporting Information Data S1). As shown in Fig. 2, there were 8031 peptides and 1125 proteins, 8257 peptides, and 1135 proteins in the male or female datasets, respectively. Of those, 3099 (23.5%) peptides and 619 (37.7%) proteins were found in both datasets. Moreover, there was no significant difference between male and female datasets in distribution of molecular mass, isoelectric point (p*I*), and predicted cellular localizations (Supporting Information Fig. S2).

3.1.3 Urinary proteomes from four different separations

Among the 13 191 identified peptides, 225 (1.7%) were found in all four datasets, 632 (4.8%) in three of the four datasets, 1542 (11.7%) in two of the four datasets, and the remaining 10 792 (81.8%) in only one of the four datasets (Fig. 3A). Of the 1641 identified proteins, 119 (7.3%) were found in all four datasets, 218 (13.3%) in three of the four datasets, 229 (13.9%) in two of the four datasets, and the remaining 1075 (65.5%) in only one of the four datasets (Fig. 3B).

3.1.4 Comparison with the MAPU urinary proteome

So far, MAPU, one of the largest datasets, is derived from the work of Adachi et al. [10, 22]. They integrated five datasets from gel or gel-free methods and reported 1543 proteins in

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Table 1. Experimental conditions and statistics on database searches constructed by means of four different separation methods

	1D SDS-PAGE	Reverse phase HPLC	p*I*-based peptide separation	2D-LC
Urinary protein	200 μg	200 μg	600 μg	200 μg
Albumin removal	+	+	+	+
Protein separation	12% Bis-Tris 1D gel	mRP-C18 column	−	−
Peptide separation	−	−	OFFGEL 3100	SCX + RP 2-DLC
Number of fraction	18	22	24	12
Digestion	In-gel	In-solution	In-solution	In-solution
Denaturant	−	6 mol/L urea + 2 mol/L thiourea		
Identified IT-MS2 spectra	29 576	24 380	45 617	13 133
Number of unique peptides	4485	3469	6065	2537
Number of identified proteins	688	580	855	540
Total number of unique peptides		13 191		
Total number of identified proteins		1641		

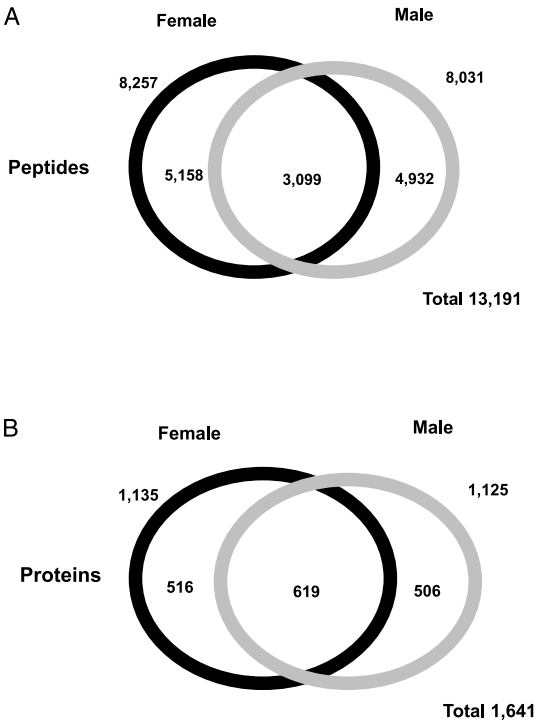

Figure 2. Comparison of the proteins identified in male and female urine pools. (A) Shared and not shared peptides. Numbers represent the several different peptides in the respective overlapping and not overlapping areas; (B) shared and not shared proteins. Numbers represent the various distinct proteins in the respective overlapping and not overlapping areas.

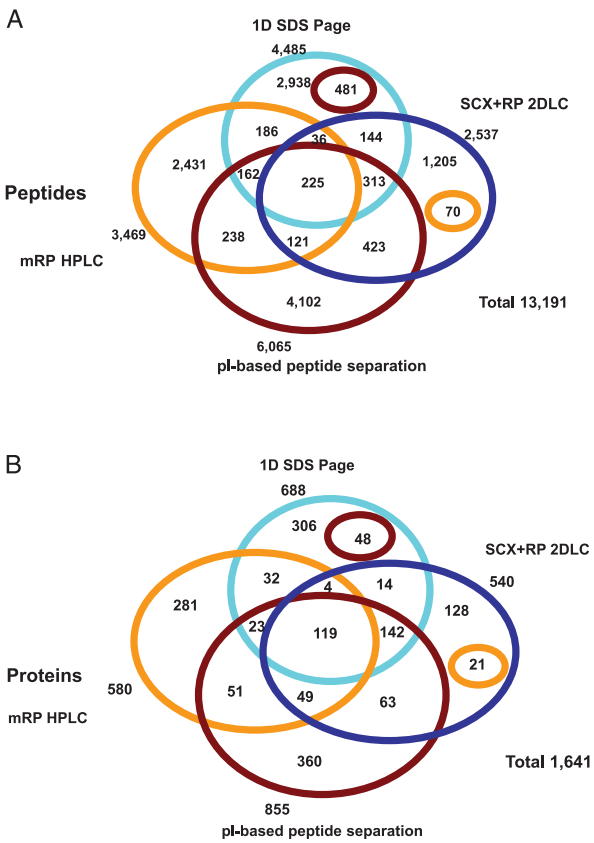

Figure 3. Comparison of the identified urinary proteins by means of 2D-LC, OFFGEL, mRP, and 1D SDS-PAGE. (A) Shared and not shared peptides. Numbers represent the various distinct peptides in the respective overlapping and not overlapping areas. (B) Shared and not shared. Numbers represent the several different proteins in the respective overlapping and not overlapping areas.

urine. The overlapping of the urinary peptides and proteins identified in MAPU and in our urinary proteome database is shown in Fig. 4. A total of 3530 (27.8%) peptides and 804 (52.1%) proteins were present in both datasets. Following GO analysis, a similarly functional category was shared between MAPUs and our urinary proteome dataset. The detailed GO analysis results are reported in Supporting Information Fig. S3 and Data S2. We analyzed the protein GO clusters of MAPUs and our urinary proteome database with ClueGO. The results showed that about 95% common terms cluster and about 5% specific terms cluster (Fig. 5).

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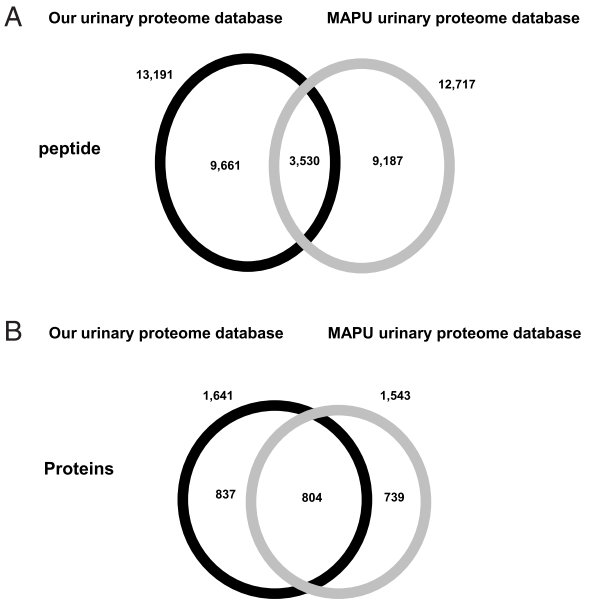

Figure 4. Relationship between the MAPU urinary proteome database and our urinary proteome database. Numbers of shared peptides or proteins are shown for our urinary proteome database (in black), and the MAPU urinary proteome database (in grey). (A) Numbers of shared peptides in the respective overlapping and not overlapping areas. (B) Numbers of shared proteins in the respective overlapping and not overlapping areas.

3.2 A stable set of urinary proteins in a healthy human population

3.2.1 Control of urinary proteome variations related to different individuals and ages

We collected morning urine samples from a total of 200 persons thrice over 4 months (at 0, 2, and 4 months). These 100 males and 100 females were healthy with normally functioning kidney and their ages ranged between 18 and 22 years. Each time, immediately after urine collection, we pooled the 100 males' and 100 females' urine samples, respectively. Pooling the samples increased the abundance of common proteins while decreasing that of individual-specific urinary proteins; thus, the interindividual variations are significantly reduced.

3.2.2 Control of urinary proteome variations related to different genders and times

The abundance-evaluation approach applied in this study is semiquantitative, and it can only be applied to proteins of a relatively high abundance. The criterion of high-abundance protein was arbitrarily set as unique peptides of >4 numbers and spectra >10 numbers in each dataset. In the OFFGEL group, a total of 101 (Supporting Information Data S3) and 127 (Supporting Information Data S4) common abundant proteins with a median abundance variation 0.128 and 0.105 were selected in the male and female subgroups, respectively. Further intergender variation analysis showed that 89 gender-common abundant proteins had a median abundance variation of 0.229 (Supporting Information Data S5). In the mRP group, 78 (Supporting Information Data S6) and 54 (Supporting Information Data S7) common abundant proteins with median abundance variations of 0.102 and 0.112 were selected in the male and female subgroups, respectively. Further intergender variation analysis showed that 37 gender-common abundant proteins had a median abundance variation of 0.269 (Supporting Information Data S8). After removing the urinary proteins with more than 0.25 interday or intergender variation, a total of 70 proteins could be selected as potential common abundant proteins in a healthy population. Among these 70 proteins, 57 proteins were present in the OFFGEL dataset and 21 proteins in the mRP dataset (Supporting Information Data S9).

3.2.3. Validation of the common urinary proteome in a healthy population

To prove that the 70 candidate proteins are indeed common urinary proteins in a healthy population, we compared these proteins with the previously published five largest urine proteomes [10–14]. Among the 70 candidate proteins, 70 (100%) were found in four datasets [10–14], and 66 (94.3%) were present in all five datasets, excepting the CADM4, KLK1, CD27, and UBC proteins. Furthermore, we compared these 66 proteins with the core urinary proteome reported by Nagarjuna et al. [14]. Thus, a total of 56 proteins obtained as common abundant urinary proteins in a healthy population (Table 2).

3.3 Validation of intact proteins

Since urinary protein fragments and polypeptides might be mistakenly taken as intact proteins, we reduced the possibility of such an erroneous identification concerning the 56 proteins by comparing in-gel urinary proteome datasets. The criterion of high-abundance protein was arbitrarily set as unique peptides of >4 numbers and spectra of >30 numbers. A total of 38 abundant proteins were found in the gel bands corresponding to the M_r's of the intact forms (Supporting Information Data S10). These 38 proteins were also found in the SDS-PAGE dataset reported by Adachi et al. [10]. To further confirm these intact proteins, we compared the 38 proteins with a urinary proteome based on 2DE gels obtained by combining the urinary proteome datasets of Jisun Oh et al. [18] and Rember Pieper et al. [19]. A total of 18 common urinary intact proteins and three protein fragments (i.e. ITIH4, CP, and HSPG2) appeared as distinct spots in 2DE gels [18]. The 18 intact proteins were identified as a stable panel of urinary proteins typical of a healthy population.

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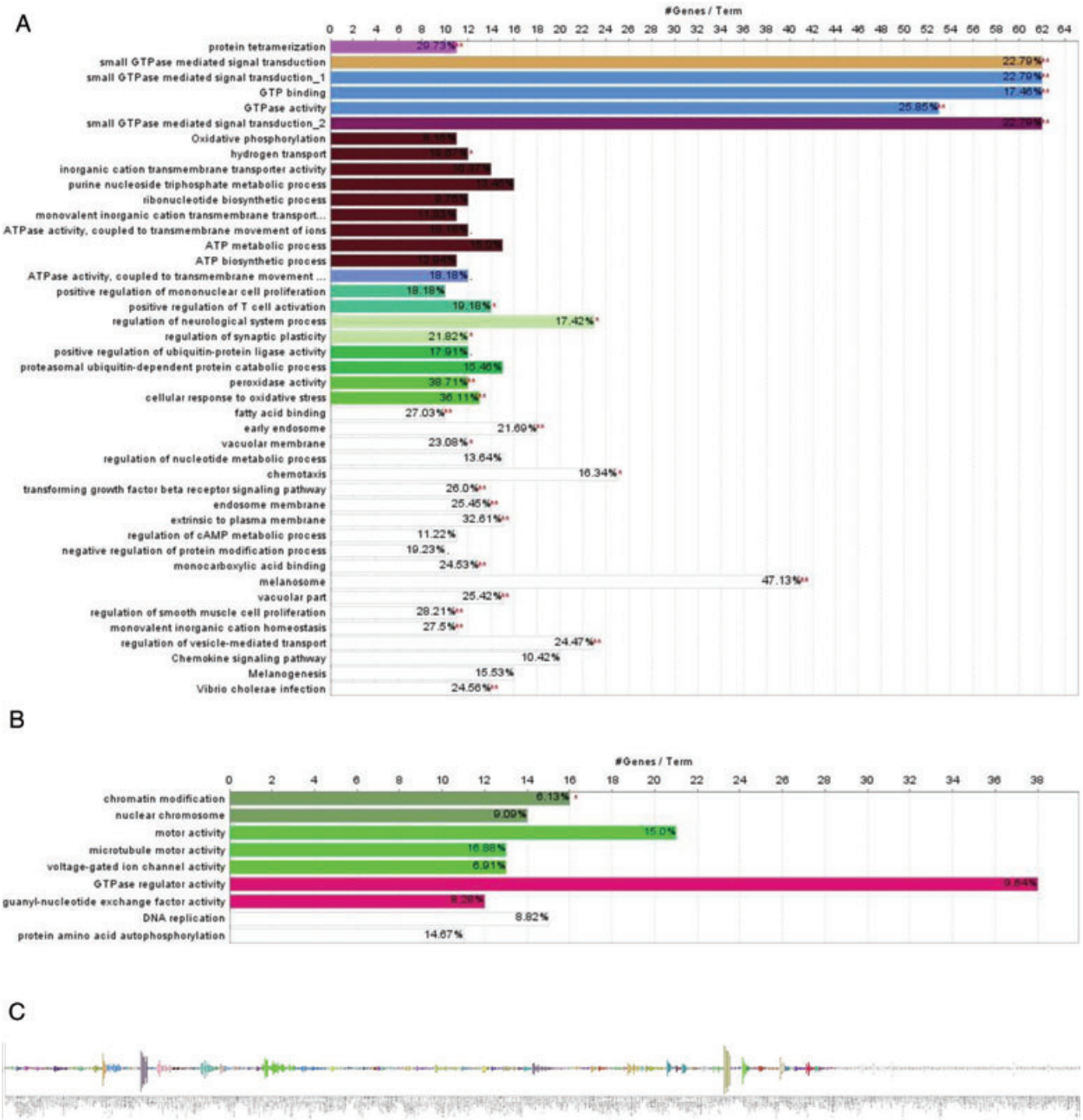

Figure 5. Gene Ontology (GO) term comparison between MAPU urinary proteome database and our urinary proteome database. All results were created using the ClueGO v. 1.2 software. Evidence codes used GO_Biological_Process_12.10.2009, GO_Cellular_Component_12.10.2009, and GO_Molecular_Function_12.10.2009. (A) Terms specific for MAPU urinary proteome database: 43. (B) Terms specific for our urinary proteome database: 9. (C) Terms common to both MAPU and our urinary proteome databases (A high-resolution version of this figure can be found in the Supporting Information).

3.4 Validation of the 18 common urinary intact proteins by ELISA

To determine the fluctuation levels of these 18 proteins in healthy individuals, we further performed ELISA assays on the urine samples from eight healthy donors (four males and four females) taken five times over 2 months (i.e. once every 2 weeks). The fluctuations of the concentration levels of COL6A1, GC, RBP4, DNAse1, GAA, SERPING1, CTSD, KNG1, UMOD, PIGR, AHSG, A1BG, AMBP, AZGP1, PTGDS, HPX, CD14, and ORM1 proteins in urine samples are reported in Table 5. The individual CV values for these 18 proteins ranged between 0.088 and 0.91 and the interday variations were the major individual sources of variation (Fig. 6).

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Table 2. The common abundant urinary proteins identified in a human healthy population

Accession_number	Protein MW	Protein p*I*	Entry_name
IPI00022895	54 272.8	5.58	A1BG alpha-1B-glycoprotein precursor
IPI00021440	41 793.1	5.31	ACTG1 actin, cytoplasmic 2
IPI00022431	39 324.9	5.43	AHSG alpha-2-HS-glycoprotein precursor
IPI00022426	38 999.7	5.95	AMBP protein precursor
IPI00025476	57 707.2	6.6	AMY1B
IPI00221224	109 540.1	5.31	ANPEP aminopeptidase N
IPI00298828	38 298.4	8.34	APOH beta-2-glycoprotein 1 precursor
IPI00166729	34 258.9	5.71	AZGP1 alpha-2-glycoprotein 1, zinc
IPI00004101	44 998.7	6.58	BHMT betaine-homocysteine S-methyltransferase 1
IPI00744685	65 050.9	6.21	BTD uncharacterized protein BTD (fragment)
IPI00783987	187 149.1	6.02	C3 complement C3 precursor (fragment)
IPI00654875	192 794.5	6.74	C4B complement C4-B precursor
IPI00176427	42 785.5	5.92	CADM4 cell adhesion molecule 4 precursor
IPI00029260	40 076.4	5.84	CD14 monocyte differentiation antigen CD14 precursor
IPI00002435	29 156.6	8.03	CD27 antigen precursor
IPI00305064	81 554	5.13	CD44 isoform CD44 of CD44 antigen precursor
IPI00099670	79 667.4	5.13	CEL carboxyl ester lipase precursor
IPI00400826	57 832.9	6.25	CLU clusterin isoform 1
IPI00329573	333 148.3	5.38	COL12A1 isoform 1 of collagen alpha-1(XII) chain precursor
IPI00291136	108 529.9	5.26	COL6A1 collagen alpha-1(VI) chain precursor
IPI00017601	122 205.8	5.44	CP ceruloplasmin precursor
IPI00556665	55 607	6.57	CSF1 colony stimulating factor 1 isoform a variant (fragment)
IPI00019954	16 511.2	8.32	CST6 cystatin-M precursor
IPI00011229	44 552.5	6.1	CTSD cathepsin D precursor
IPI00889603	398 738.6	5.13	CUBN cubilin
IPI00031065	31 433.9	4.71	DNASE1 deoxyribonuclease-1 precursor
IPI00025846	99 962.2	5.18	DSC2 isoform 2 A of desmocollin-2 precursor
IPI00000073	133 947.1	5.55	EGF pro-epidermal growth factor precursor
IPI00019568	70 037.3	5.64	F2 prothrombin precursor (fragment)
IPI00073772	36 814.7	6.54	FBP1 fructose-1,6-bisphosphatase 1
IPI00640044	32 690.7	8.36	FCGR3 A Fc fragment of IgG, low-affinity IIIa, receptor for
IPI00883772	105 324.4	5.62	GAA acid alpha-glucosidase preproprotein
IPI00555812	52 964	5.4	GC vitamin D-binding protein precursor
IPI00441344	76 091.3	6.1	GLB1 isoform 1 of beta-galactosidase precursor
IPI00012102	62 082.5	8.6	GNS N-acetylglucosamine-6-sulfatase precursor
IPI00102300	67 309.3	9.11	GP6 isoform 3 of platelet glycoprotein VI precursor
IPI00045512	613 707.5	6.04	HMCN1 hemicentin 1
IPI00022488	51 676.7	6.55	HPX hemopexin precursor
IPI00024284	468 827.8	6.06	HSPG2 basement membrane-specific heparan sulfate proteoglycan core protein precursor
IPI00016915	29 130.5	8.25	IGFBP7 insulin-like growth factor-binding protein 7 precursor
IPI00829944	51 254.2	7.88	IGHG1 IGHG1 protein
IPI00294193	103 325.9	6.51	ITIH4 isoform 1 of interalpha-trypsin inhibitor heavy chain H4 precursor
IPI00304808	28 889.7	4.62	KLK1 kallikrein-1 precursor
IPI00797833	47 901.5	6.29	KNG1 kininogen 1
IPI00743064	22 902.5	9.02	LCN2 uncharacterized protein LCN2
IPI00023673	65 331.4	5.13	LGALS3BP galectin-3-binding protein precursor
IPI00024292	521 961.3	4.89	LRP2 low-density lipoprotein receptor-related protein 2 precursor
IPI00294713	75 733.9	5.47	MASP2 isoform 1 of Mannan-binding lectin serine protease 2 precursor
IPI00015525	104 417	5.5	MMRN2 multimerin-2 precursor
IPI00008787	82 167	6.1	NAGLU alpha-N-acetylglucosaminidase precursor
IPI00009901	14 478.6	5.1	NUTF2 nuclear transport factor 2
IPI00884926	23 539.7	5.02	ORM1 orosomucoid 1 precursor
IPI00021085	21 730.9	8.92	PGLYRP1 peptidoglycan recognition protein precursor
IPI00004573	83 284	5.59	PIGR polymeric immunoglobulin receptor precursor
IPI00009276	30 715.3	8.8	PROCR endothelial protein C receptor precursor
IPI00012540	97 202.6	6.97	PROM1 prominin-1 precursor
IPI00219825	61 693.2	5.12	PSAP prosaposin
IPI00013179	21 028.9	7.65	PTGDS prostaglandin-H2 D-isomerase precursor
IPI00022420	23 010.1	5.76	RBP4 plasma retinol-binding protein precursor
IPI00009027	18 731.1	5.65	REG1 A lithostathine 1 alpha precursor
IPI00014048	17 644.4	9.1	RNASE1 ribonuclease pancreatic precursor
IPI00019449	18 354.3	9.1	RNASE2 nonsecretory ribonuclease precursor
IPI00007047	10 834.6	6.51	S100A8 protein S100-A8
IPI00027462	13 242.1	5.71	S100A9 protein S100-A9
IPI00170635	27 039.2	7	SECTM1 secreted and transmembrane protein 1 precursor
IPI00007221	45 702	9.3	SERPINA5 plasma serine protease inhibitor precursor
IPI00291866	55 154.5	6.09	SERPING1 plasma protease C1 inhibitor precursor
IPI00021000	35 422.9	4.37	SPP1 isoform A of osteopontin precursor
IPI00793330	12 252.2	5.85	UBC
IPI00640271	73 571.5	5.08	UMOD 74 kDa protein
IPI00291488	12 993.1	4.69	WFDC2 isoform 1 of WAP four-disulfide core domain protein 2 precursor

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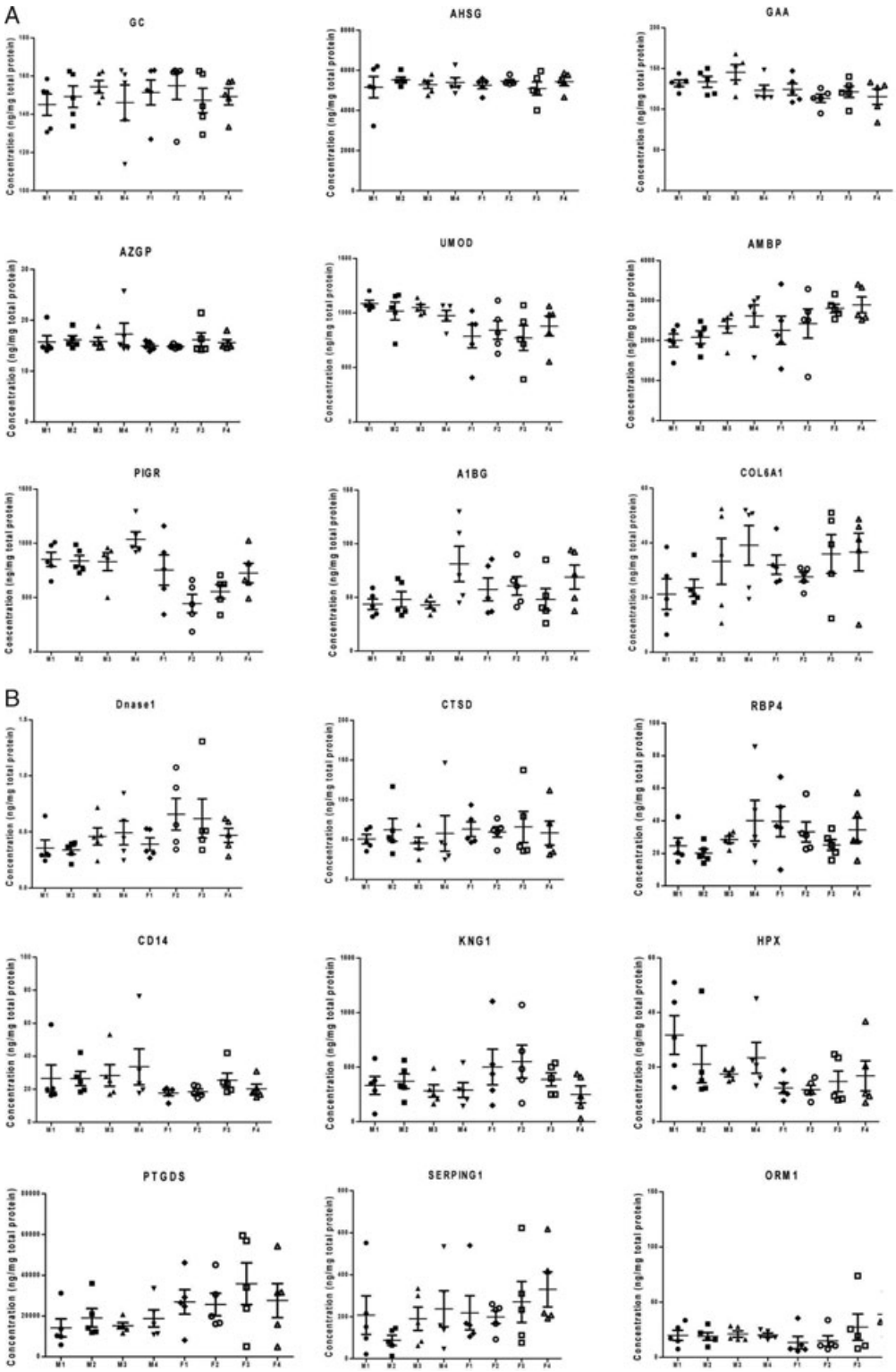

Figure 6. ELISA scatter plots of 18 urinary protein levels for five time points during 2 months observed in eight healthy donors (four males and four females). A total of 40 urinary protein samples, which were collected five times over 2 months from four male and four female healthy donors, were assayed for the plotted proteins. All values are adjusted for total protein levels. The horizontal axis legend represents: M, male; F, female.

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Table 3. Characterization of a panel of 18 intact common urinary proteins

IPI ID	Official symbol	Localization	Genetic disease	Glycosylation
IPI00291136	COL6A1	Extracellular (GO:0005576)	Bethlem myopathy	Null
IPI00742696	GC	Extracellular (GO:0005576)	GC1/GC2 polymorphism	Null
IPI00022420	RBP4	Extracellular (GO:0005576)	Retinol-binding protein deficiency	Null
IPI00031065	DNASE1	Extracellular (GO:0005576)	Systemic lupus erythematosus, susceptibility to	Null
IPI00293088	GAA	Lysosome (GO:0005764)	Acid alpha-glucosidase, allele 4	Glycosylation
IPI00291866	SERPING1	Extracellular (GO:0005576)	Angioedema, hereditary, autosomal recessive	Glycosylation
IPI00011229	CTSD	Extracellular (GO:0005576)	Ceroid lipofuscinosis, neuronal, 10	Glycosylation
IPI00797833	KNG1	Extracellular (GO:0005576)	Fitzgerald trait	Glycosylation
IPI00640271	UMOD	Extracellular (GO:0005576)	Glomerulocystic kidney disease with hyperuricemia and isosthenuria	Glycosylation
IPI00004573	PIGR	Extracellular (GO:0005576)	IgA nephropathy, susceptibility to	Glycosylation
IPI00022431	AHSG	Extracellular (GO:0005576)	Leanness, susceptibility to	Glycosylation
IPI00022895	A1BG	Extracellular (GO:0005576)	Null	Glycosylation
IPI00022426	AMBP	Extracellular (GO:0005576)	Null	Glycosylation
IPI00166729	AZGP1	Extracellular (GO:0005576)	Null	Glycosylation
IPI00013179	PTGDS	Extracellular (GO:0005576)	Null	Glycosylation
IPI00022488	HPX	Extracellular (GO:0005576)	Null	Glycosylation
IPI00029260	CD14	Extracellular (GO:0005576)	Null	Glycosylation
IPI00884926	ORM1	Extracellular (GO:0005576)	Orosomucoid polymorphism	Glycosylation

Table 4. The relationship between a panel of 18 common urinary proteins and diseases

ORM1[a,b]	Acute kidney injury after cardiac surgery in children [44]. Diabetic nephropathy [9]. Interstitial cystitis/painful bladder syndrome [45].
AHSG[a,b]	Acute kidney injury [6, 7]. Vascular calcifications [46, 47]. Breast cancer [48].
AMBP[a,b]	IgA nephropathy[49]. Acute hepatitis E [50]. Acute kidney injury after cardiac surgery in children [44]. Renal toxicity in trichloroethylene-exposed persons [51]. Diabetes mellitus type 2 [30].
AZGP1[a,b]	Diabetes mellitus type 2 [30]. Cancer cachexia [31]. Acute kidney injury after cardiopulmonary bypass surgery [32]. Acute rejection after renal transplantation [5].
CTSD[a,b]	Renal cell carcinoma [3]. Colon adenocarcinoma [52].
CD14[a,b]	Polycystic kidney disease [53]. Benign prostatic hyperplasia [54].
HPX[a,b]	Interstitial cystitis/painful bladder syndrome [45]. Childhood HIV-associated renal diseases [55].
KNG1[a,b]	Ovarian carcinoma [56]. Kidney chronic allograft dysfunction [26].
SERPING1[a,b]	Acute kidney rejection [4].
PTGDS	Early gentamicin-induced renal damage [57]. Lupus nephritis [58]. Vascular injury in type 2 diabetes patients [59].
UMOD[a]	IgA nephropathy [25]. Kidney chronic allograft dysfunction [26]. Autosomal dominant polycystic kidney disease [27]. Acute kidney rejection [4]. Kidney stone disease [28, 29].
GC[b]	Multiple myeloma [60]. Neuromyelitis optica [61]. Acute renal allograft rejection [33]. Multiple sclerosis [62].
RBP4[a,b]	Diabetes mellitus type 2 [30]. Diabetic nephropathy [63]. Childhood HIV-associated renal diseases [55].
A1BG[a,b]	Pediatric steroid-resistant nephrotic syndrome [64].
PIGR[a,b]	
DNASE1	
COL6A1[a]	
GAA[a]	

a) Urinary exosome;
b) Plasma

3.5 Characterization of the stable set of urinary proteins

To learn the characteristics of these 18 urinary proteins, we firstly analyzed them using the HPRD database, which is a most reliable database. The typical features of such proteins, including information about HPRD ID, official symbol, glycosylation, and genetics disease, are reported in Table 3. Almost all of them are extracellular proteins, save the plasma protein GAA, and 14 of them (77.8%) can be modified via glycosylation. According to the HPRD database, 12 of 18 proteins are associated with genetic ailments. To further learn the relationships between these 18 proteins and diseases, we searched the Pub Med database and found that 14 proteins, that is excluding DNAse1, PIGR, GAA, and COL6A1, had been reported as potential disease biomarkers (Table 4). After comparing the same 18 proteins with the plasma proteome and the urinary exosomal proteome reported by Pisitkun et al. [23] and Gonzales et al. [24], we found that ten urinary proteins (i.e. A1BG, AHSG, AMBP, AZGP1, CD14, CTSD, HPX, KNG1,

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ORM1, PIGR, RBP4, and SERPING1) were presented in both plasma and urinary exosome proteomes, three urinary proteins (i.e. COL6A1, GAA, and UMOD) in the urinary exosome proteome, one urinary protein (GC) in the plasma proteome, and two urinary proteins (DNAse1 and PTGDS) only in urine (Table 4).

4 Discussion

4.1 The urinary proteome database of a human healthy population

In this study, the urinary proteome of a human healthy population was analyzed by means of four fractionation techniques (i.e. in-gel, 2D-LC, OFFGEL, and mRP) coupled with HPLC-Chip-MS/MS. The overall features of the datasets derived from the four fractionation techniques are shown in Fig. S3, and similar trends were observed among these four datasets concerning the distributions of M_r's, p*I*, and predicted cellular localizations (Supporting Information Fig. S3). This demonstrated that the intrinsic bias linked to protein identification inherent to each separation method could be minimized through the parallel use of multiple fractionation techniques. Among the four separation methods, the OFFGEL approach identified the largest number of proteins while also providing quantitative information, but required much more work and time; the in-gel approach identified the second largest number of proteins along with M_r's information, but was not suitable for appraising protein abundance.

A total of 1641 urinary proteins were identified with a high confidence (Supporting Information Data S1). Through comparing them with MAPUs data, we observed approximately 50% of protein overlap (Fig. 4 and Supporting Information Data S2) and 95% of similarity with GO functional categories (Fig. 5). Alike results obtained by further comparisons with four other urinary proteomes (data not shown). This made us confident that the data we report in this study are reliable. Due to the considerable degree of dynamic variations in the urinary proteome, it was required to further establish the existence of a stable set of urinary proteins in a human healthy population by controlling such urinary proteome changes [15].

4.2 A stable panel of urinary proteins in a human healthy population

The urinary proteome differs from one to another individual and according to the actual age [15]. In this study, the age-related variations were reduced by limiting urine sample collection to healthy donors with an age range of 18–22 years, while the interindividual variations were adequately controlled by using urine samples from a total of 200 persons (Fig. 1). The urinary proteome also differs between sexes [15]. Thus, urinary proteomes derived from 100 male and 100 female healthy donors were determined, and 619 (37.7%) proteins were found in both datasets. And there was no significant difference between male and female datasets in the distribution of M_r's, p*I*, and the predicted cellular localizations (Supporting Information Fig. S2). Furthermore, a total of 14 male-predominant and eight female-predominant stable urinary proteins (Supporting Information Data S11) were identified by comparing the male and female datasets. Among the 14 male-predominant proteins, KLK3 was confirmed to be a male-specific protein.

Since the urinary proteome changes markedly over time [15], a stable set of urinary proteins proper of a human healthy population should obtain from urine samples with low interday and intergender variations. Seventy abundant proteins were identified by us as potential candidates for a stable panel of urinary proteins typical of a healthy population with interday and intergender variations within 0.25 (Supporting Information Data S5, S8, S9). Finally, among the just mentioned 70 proteins, 56 were further confirmed to belong to a stable set of urinary proteins via comparisons with the previously published five largest urinary proteomes [10–14] (Table 2).

Since protein fragments in urine could be incorrectly identified as intact proteins by peptide identification based on MS/MS proteomics, some of the proteins in this group might have derived from the wrong identification of protein fragments. Therefore, we employed a 10 kDa cut-off ultrafiltration membrane to remove the low molecular weight polypeptides from the urine samples. Next, the 18 intact proteins were confirmed as constituting a stable urinary panel proper of a healthy population by comparisons with the urinary proteomes from in-gel datasets [10] and 2DE datasets [18, 19]. Finally, all of the 18 urinary proteins were validated with ELISA assays in samples taken from eight healthy individuals during 2 months. The individual CV values for these 18 proteins ranged between 0.088 and 0.91 (Table 5). Among them, the CV values for GC, AHSG, GAA, AZGP, UMOD, and AMBP were lower than 0.25. We surmise that these 18 urinary proteins represent a stable set proper of a healthy population.

4.3 A panel comprising 18 intact urinary proteins reflects the human good health condition

According to the HPRD database, 12 of the 18 common urinary proteins were linked to genetic diseases (Table 3). It is interesting to find that 14 of the 18 common urinary proteins were previously indicated as potential disease biomarkers (Table 4). Each of these 14 proteins was reported as a potential biomarker for multiple diseases rather than for a single disease (Table 4). For example, UMOD turned up to be a likely biomarker for IgA nephropathy [25], kidney chronic allograft dysfunction [26], autosomal dominant polycystic kidney disease [27], acute kidney rejection [4], and kidney stone disease [28, 29]. Another example was AZGP1, which was found to be

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Table 5. The fluctuation levels of the 18 common proteins in a healthy individual's urine

Official symbol	Protein concentration (ng/mg total protein) mean ± SD ($n = 40$)	CV
GC	149.70 ± 13.23	0.088
AHSG	5327.55 ± 568.21	0.107
GAA	126.10 ± 17.54	0.139
AZGP	15.70 ± 2.24	0.143
UMOD	925.86 ± 198.44	0.214
AMBP	2431.20 ± 572.60	0.236
PIGR	753.93 ± 245.44	0.326
A1BG	56.34 ± 23.74	0.421
COL6A1	31.16 ± 13.39	0.430
Dnase1	0.47 ± 0.23	0.490
CTSD	58.07 ± 28.75	0.495
RBP4	30.71 ± 15.54	0.506
CD14	24.56 ± 13.01	0.530
KNG1	370.17 ± 224.38	0.606
HPX	18.65 ± 11.48	0.615
PTGDS	22.91 ± 14.27	0.623
SERPING1	217.50 ± 163.02	0.749
ORM1	21.90 ± 19.92	0.910

a probable biomarker for diabetes mellitus type 2 [30], cancer cachexia [31], acute kidney injury after cardiopulmonary bypass surgery [32], and acute rejection after renal transplantation [5]. In addition, several of these proteins were indicated as prospective biomarkers for the same illness (Table 4). For example, AMBP, AZGP1, and RBP4 were reported to be likely biomarkers for diabetes mellitus type 2 [30]. Other examples are AZGP1 [5], SERPING1 [4], UMOD [4], and GC [33], which were reported as potential biomarkers for acute rejection after kidney transplantation. These lines of evidence indicate that the 18 common urinary proteins could, in the first instance, serve as potential biomarkers reflecting the human healthy status rather than certain diseases. It may be reasonable to assume that, as a group, these 18 proteins could indicate a human healthy condition. Abnormalities in this common urinary protein panel may awaken people's attention to an unhealthy condition. On the other hand, the rescue of a normal common urinary protein panel may indicate that a patient is healing. However, further assessments of the normal fluctuations of each single urinary protein pertaining to the panel are needed prior to any translation to clinical practice. Subsequently, intensive clinical studies would also be required to determine the ranges within which the dynamic changes in the protein panel still indicate a human healthy condition.

It is known that glycosylation changes on glycoproteins are closely associated with diseases. Notably, 14 of the 18 common urinary proteins are glycoproteins (Table 3), and 12 were reported as posttranslationally modified proteins [19]. Glycosylation changes of these proteins in the serum might contribute to diseases [34–43] (Supporting Information Table S3). Furthermore, we observed that IGHG1 protein had the same glycosylation modification in both urine and serum (data not shown). Hence, it is needed to further investigate whether glycosylative changes in these 14 common urinary glycoproteins might reveal a diseased status of the urinary system.

4.4 Concluding remarks

Our present study has allowed to identify with a high confidence not only a human urinary proteome comprising 1641 proteins, but even a reproducible panel of 18 intact proteins, which pertains to a human healthy population. This latter panel could serve as a potential biomarker indicative of healthy conditions.

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