

RESEARCH ARTICLE

Characterization of the renal cyst fluid proteome in autosomal dominant polycystic kidney disease (ADPKD) patients

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by localized autonomous cellular proliferation, fluid accumulation within the cysts, and intraparenchymal fibrosis of the kidney. Little is known about the cyst fluid's protein composition. We hypothesized that the complex collection of cyst fluid proteins (cyst fluid proteome) plays a major role in cyst formation/maintenance and contains yet unknown diagnostic and mechanistic features that are common to all forms of PKD. We analyzed five kidney cyst fluids from four patients with ADPKD. Tryptic peptides from plasma-protein immunodepleted (ProteoPrep[®]) and undepleted cyst fluid samples were analyzed by LC-MS/MS. Proteins were identified by SEQUEST[™] and validated *via* the Trans-Proteomic Pipeline; 391 proteins were identified with >90% confidence; 251 of them in undepleted and 362 in immunodepleted samples. Immunodepletion removed >94% of the cyst fluid protein. A surprisingly large and functionally diverse number of proteins common to most cysts were identified. These proteins may be of mechanistic interest and include Ig γ , κ , and fragments; complement components; vitronectin; orosomucoid; prostaglandin D2 synthase; vitamin D-binding protein; clusterin; SERPIN family proteins; hemo-pexin; and fetuin-A. Additionally, these results suggest that further prefractionation and enhanced chromatographic separation of tryptic peptides is likely to expose an even greater number of relevant proteins.

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Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; ARPKD, autosomal recessive polycystic kidney disease; IPI, International Protein Index; PKD, polycystic kidney disease; TPP, Trans-Proteomic Pipeline

1 Introduction

Polycystic kidney disease (PKD) is a common genetic disorder that results in bilateral renal enlargement due to the development of fluid filled cysts. This disease afflicts 5–10% of all end-stage renal disease patients in the US and its pathology is associated with hypertension and other extra-renal complications, rendering it a considerable public health burden [1]. Two forms of PKD are diagnosed, autosomal dominant PKD (ADPKD) and autosomal recessive

PKD (ARPKD). ADPKD results from mutations in a set of genes that encode the proteins polycystin-1 (PKD1) and polycystin-2 (PKD2) [2, 3], two integral membrane proteins localized in the renal cilia [4]. ADPKD (PKD1) is much more common, affects approximately 1 in 800 in the human population, and is usually diagnosed after the fourth decade of life. ARPKD is a more severe disease that afflicts neonates and children. Renal failure in ADPKD is due to the infiltration of the functional parenchyma with monocytes and fibroblasts that results in renal fibrosis [5].

Recent research has focused on the genetic defects and processes involved in cyst formation in PKD. However, little is known about the growth and maintenance of renal cysts and how these processes might relate to cyst fluid constituents. Previous studies have identified a few of the proteins present in the renal cyst fluid: EGF [6–8], TGF- α [7, 9], amphiregulin (AR) and heparin-binding (HB)-EGF [7], laminin fragments [10], nanobacterial constituents [11], α 1 antitrypsin, prealbumin, hemopexin, α 1 antichymotrypsin, transferrin, IgG, IgA, and alanine aminotransferase [12]. Nevertheless, the complex protein constituency of cyst fluid remains unknown.

It is our hypothesis that the collection of cyst fluid proteins (cyst fluid proteome) is diverse and complex, that some if not many of these proteins may play a major role in liver cyst development and maintenance, and that they embody both diagnostic and mechanistic features that may be common to all forms of cystic disease, but are not yet known. An effective approach to test this hypothesis is mass spectrometric-based proteomic analysis. This approach enables one to identify the proteins contained in complex biological samples and, using various additional spectral data analyses [13], can provide relative quantitation from individuals whose protein synthesis or degradation rates are affected by the state of the organism or a tissue. Proteins that are present or absent in a disease state, such as PKD, can be used as markers for mechanistic explanation, diagnoses, or potential targets for therapeutic intervention.

The renal cyst fluid proteome is of particular interest because it has been shown to have proliferative and secretory properties that are likely to initiate cyst growth and maintain the cysts [14, 15]. Gattone *et al.* [16] described mRNA misexpression in organs involved in the murine BALB/c-cpk/cpk model of ARPKD. These mRNAs are known to be involved in proliferation, apoptosis, differentiation, and/or extracellular matrix (ECM). While some of the factors have been identified, it is likely that additional components of the cyst fluid, when identified, will provide further information on the elements that cause cyst formation and growth. Proteins associated with differentiation and dedifferentiation processes, specifically those related to secretory pathways and ECM, may reflect alterations in polarized protein sorting/trafficking [17, 18] and be involved in renal cyst development [19]. It is likely that these will be found in the cyst fluid.

The most widely used proteomic method for assessing protein expression globally is “bottom-up” proteomics. In this strategy, proteins in a body fluid or cell/tissue extract are digested proteolytically and the resulting complex peptide mixture is separated by unidimensional or multidimensional LC coupled to MS/MS for the identification of all the proteins present in the original sample. For the first time, we present a comprehensive analysis of the proteins present in human renal cyst fluid, discuss their relevance in terms of cyst growth and maintenance, and suggest additional proteomic strategies for further study.

2 Materials and methods

2.1 Materials

TFA, iodoethanol, triethylphosphine (TEP), and ammonium bicarbonate were purchased from Sigma–Aldrich (St. Louis MO, USA). Acetone was obtained from Fisher Scientific (Philadelphia, PA, USA). ACN and MS grade water were purchased from EMD Chemicals (Gibbstown, NJ, USA). Modified sequencing grade porcine trypsin was obtained from Princeton Separations (Freehold, NJ, USA). The ProteoPrep[®] 20 plasma immunodepletion kit was purchased from Sigma–Aldrich. The 2-D Quant Kit was purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.2 Clinical specimens

Sample management of de-identified samples was performed according to the bioethical recommendations of the Institutional Review Board. Human renal cyst fluid samples were obtained from patients by needle aspiration, immediately frozen in liquid nitrogen, and stored at -80°C until use. The diagnosis of ADPKD was made clinically. More clinical specimen information is provided in Table 1.

2.3 Depletion of high-abundance plasma proteins

Preliminary sample evaluation revealed an excessive abundance of albumin and other plasma proteins in the cyst fluid. As discussed later in the paper, this necessitated removal of these proteins to facilitate the identification of low-abundance proteins. Therefore, a 2 mL aliquot of each sample was centrifuged at 3000 rpm, 4°C for 10 min. The supernatant concentration was determined by the Bradford protein assay [20]. A 500 μL aliquot of each centrifuged sample was precipitated by addition of nine volumes of ice-cold acetone containing 10% TFA v/v and immediately mixed by gentle vortexing. The mixture was incubated overnight at 4°C and centrifuged at 4500 rpm, 4°C for 20 min. The precipitate was mixed with 2 mL ice-cold acetone, incubated at 4°C for 30 min and centrifuged as above. The precipitate was air-dried by SpeedVac and 1 mL of 4 M urea was added to dis-

Table 1. Sample information

Sample	Gender	Age	Cyst diam. (mm)	Cyst fluid description	Volume (mL)	Concentration (mg/mL)
CF01	Female	39	22.5	Cloudy, brown	6.0	1.58
CF02	Female	47	18.8	Clear, yellow	3.5	41.36
CF03	Female	59	21.2	Clear, yellow	5.0	27.79
CF04 ^{a)}	Female	47	17.5	Clear, yellow	2.8	22.34
CF05	Male	50	39.4	Clear, yellow	32.0	23.93

a) same patient as CF02, different cyst.

solve the precipitated sample. Again, protein concentration was determined by the Bradford assay.

Depletion of high-abundance plasma proteins was performed as described in the ProteoPrep 20 User Guide. Briefly, 100 μ L of diluted precipitated sample was placed on the equilibrated spin column and incubated at room temperature for 20 min. The spin column and collection tube were then centrifuged at 5000 rpm for 30 s at room temperature. The flow through volume was saved in the collection tube. The spin column was then washed twice with 100 μ L of equilibration buffer. Total fluid collection was 300 μ L. The proteins bound to the column were eluted with 2 mL of elution solution and 1.5 mL of the first elution was collected. Each sample was processed as four technical replicates. The flow through volumes were pooled and dried *via* SpeedVac. These protein samples are subsequently referred to as “depleted samples.” Afterward, the bound proteins were eluted and their eluants were pooled and the concentration of this pooled solution was determined by the 2-D Quant Kit assay.

2.4 Protein reduction, alkylation, and digestion

2.4.1 Undepleted samples

Proteins in 200 μ L of the diluted, TFA/acetone precipitated cyst fluid samples were reduced and alkylated by TEP and iodoethanol as described by Hale *et al.* [21]. Briefly, 200 μ L of the reduction/alkylation cocktail was added to the protein solution. The sample was incubated at 37°C for 90 min, dried by SpeedVac, and reconstituted with 100 μ L of 100 mM NH_4HCO_3 at pH 8.0. A 150 μ L aliquot of a 20 μ g/mL trypsin solution was added to the sample and incubated at 37°C for 3 h after which another 150 μ L of trypsin was added, and the solution incubated at 37°C for 3 h.

2.4.2 Depleted samples

CF01 was reduced, alkylated, and digested as above. Due to their lower protein content, the other samples (CF02–CF05) were reconstituted in 100 μ L of 4 M urea, reduced/alkylated

by 100 μ L of the cocktail, dried, reconstituted with 50 μ L of NH_4HCO_3 , and digested by two 75 μ L additions of trypsin solution. Finally 50 μ L of 100 mM NH_4HCO_3 was added to each digested depleted sample except CF01.

2.5 LC-MS/MS

The digested samples were analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). Tryptic peptides were injected onto the C18 microbore RP column (Zorbax SB-C18, 1.0 mm \times 150 mm) at a flow rate of 50 μ L/min. The mobile phases A, B, and C were 0.1% formic acid in water, 50% ACN with 0.1% formic acid in water, and 80% ACN with 0.1% formic acid in water, respectively. The gradient elution profile was as follows: 10% B (90% A) for 5 min; 10–95% B (90–5% A) for 120 min; 100% C for 5 min; and 10% B (90% A) for 12 min. The data were collected in the “Triple-Play” (MS scan, Zoom scan, and MS/MS scan) mode with the ESI interface using a normalized collision energy of 35%. Dynamic exclusion settings were set to repeat count 1, repeat duration 30 s, exclusion duration 120 s, and exclusion mass width 0.75 m/z (low) and 2.0 m/z (high).

2.6 Data analysis

The acquired data were searched against the International Protein Index (IPI) human database (ipi.HUMAN.v3.34) using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). General parameters were set to: peptide tolerance 2.0 amu, fragment ion tolerance 1.0 amu, enzyme limits set as “fully enzymatic – cleaves at both ends”, and missed cleavage sites set at 2. The searched peptides and proteins were validated by PeptideProphet [22] and ProteinProphet [23] in the Trans-Proteomic Pipeline (TPP, v. 3.3.0) (<http://tools.proteomecenter.org/software.php>). Each protein identification was reported and compared using an in-house developed platform named LASPAP (LArge-Scale Shotgun Proteomics data Analysis Platform). Gene ontology analysis was completed using the Generic GO Term Finder [24] developed by the Bioinformatics Group at the Lewis-Sigler Institute at Princeton (<http://go.princeton.edu>).

3 Results and discussion

3.1 Sample preparation

In view of our preliminary results that indicated the predominance in cyst fluid of albumin and other prominent plasma proteins in cyst fluid and our interest in desalting the fluid prior to proteolysis/LC, we employed a two-stage sample cleanup strategy. This included TFA/acetone precipitation followed by immunodepletion. Protein precipitation is typically used to remove salts and concentrate proteins. However, it is also capable of depleting at least a portion of the albumin contained in a sample. In a previous study, TCA, HCl, CH₃COOH, and H₃PO₄ were added to acetone individually to determine the most effective precipitation solution. In addition to providing excellent precipitation, TCA/acetone also was found to be the most efficient method for albumin removal [25]. Though TFA/acetone was not evaluated in that experiment, we tested it in the present study. Our results indicated that 24.2, 26.8, 47.4, 45.6, and 34.0% of the original protein (albumin) was depleted from each sample (CF01–CF05, respectively) using nine volumes of TFA/acetone. The low-abundance protein concentration increased from 3.0% in unprecipitated CF01 to 21.0% after precipitation. These data confirm that the TFA/acetone approach, like TCA/acetone, is an efficient way to remove albumin, as applied in this study. Hereafter, “low-abundance proteins” will refer to those proteins present in the cyst fluid after removal of albumin and the other high-abundance plasma proteins by TFA/acetone precipitation and immunodepletion.

In addition to albumin, both PKD renal cyst fluid and plasma contain large fractions of IgG, apolipoproteins, trypsin inhibitors, and other high-abundance proteins. Therefore, the depletion of the high-abundance plasma proteins in the cystic fluid was deemed a prerequisite to increase our analytical “depth of field.” The ProteoPrep 20 plasma immunodepletion kit [26], which removes 20 of the top high-abundance proteins using conventional antibodies

coupled to small recombinant immunoaffinity ligands, was applied in this experiment to remove them from the cyst fluid. After depletion, protein assay confirmed that 79.0, 99.0, 98.4, 97.9, and 97.9% of the proteins in the original samples (CF01, CF02, CF03, CF04, and CF05, respectively) were removed by the immunodepletion column. These results clearly emphasize the necessity of high-abundance protein depletion to facilitate identification of the remaining cyst fluid proteome.

The depletion of high-abundance proteins, that can mask the presence of low-abundance proteins, increases the relative concentration of low-abundance proteins and therefore improves detection sensitivity [27]. As our data in Fig. 1 demonstrate, the number of peptides identified with $\geq 90\%$ confidence for each sample (technical replicates A and B) increased dramatically after immunodepletion. Likewise, proteins identified from these peptides reveal the presence of 112, 71, 60, 64, and 72 low-abundance proteins in the depleted samples CF01–CF05, respectively, compared to 56, 45, 29, 38, and 38 from the undepleted samples (see Fig. 2).

3.2 Protein identification

The peptides and proteins identified by SEQUEST were evaluated with the PeptideProphet and ProteinProphet to determine their identification probabilities (Table 2). The validated proteins from the various samples were then reported and compared with LASPAP. The complete validated protein identification list and the protein comparisons between all the samples appear in the Supporting Information. Figure 3 illustrates a total and low-abundance protein comparison between the depleted and undepleted samples, combining data from all individuals. A total of 391 proteins were identified with $\geq 90\%$ confidence. Of those, 362 were from the depleted samples and 251 from the undepleted samples (Fig. 3A). Low-abundance proteins comprised 225 out of the 391 identified proteins (Fig. 3B). Among these, 199 were from the depleted samples and 106 from the undepleted samples.

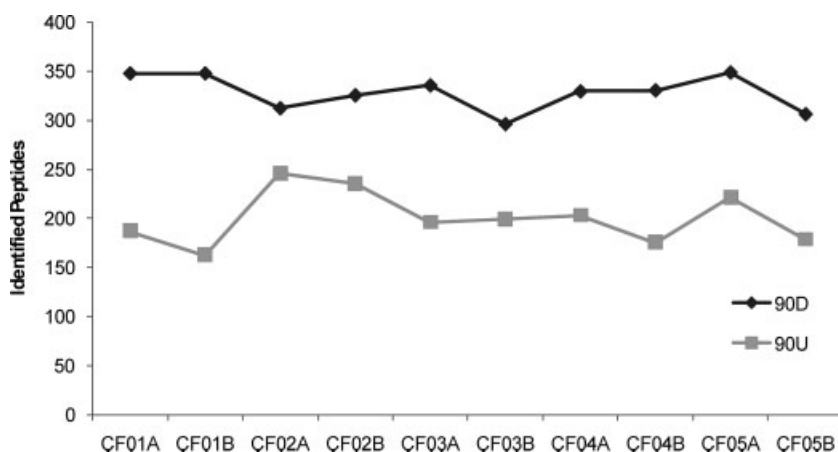


Figure 1. Comparison of peptides identified with $\geq 90\%$ confidence between the depleted and undepleted samples. Each sample underwent two injections (A and B). 90D refers to the depleted samples and 90U to the undepleted samples. These data demonstrate the utility of depleting the 20 most abundant plasma proteins from the sample, as significantly more peptides were identified after immunodepletion.

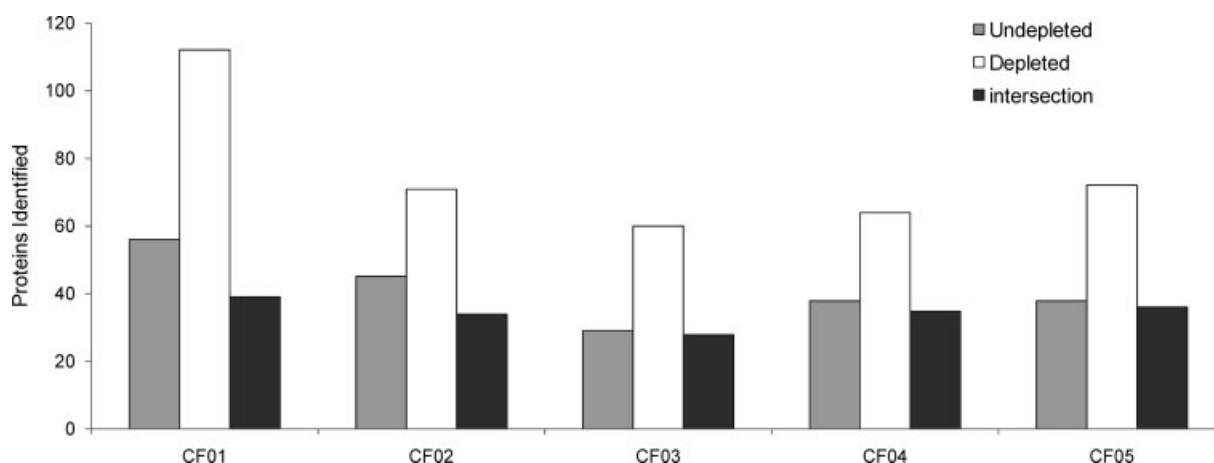


Figure 2. The “low-abundance protein” distribution comparison for each individual sample, where proteins were detected in two technical replicates. Low-abundance proteins are those present in the cyst fluid after removal of albumin and the other high-abundance plasma proteins by TFA/acetone precipitation and immunodepletion. 112, 71, 60, 64, and 72 low-abundance proteins were detected in the depleted samples CF01–CF05, respectively, compared to 56, 45, 29, 38, and 38 from the undepleted samples, an improvement in detection sensitivity after depletion. The intersection of the datasets for each sample illustrates the relative number of “medium-abundance proteins” present in the samples, *e.g.*, those whose detection was not hindered by high-abundance proteins in the undepleted sample.

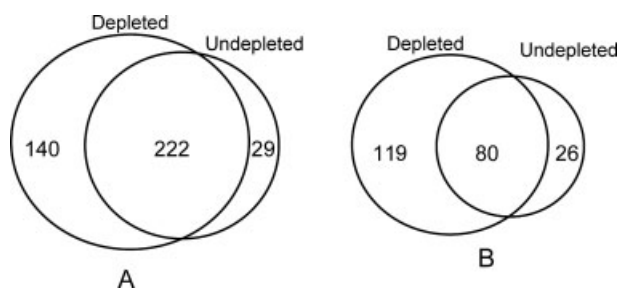


Figure 3. Venn diagrams of the relationship between sets of proteins identified in depleted samples and undepleted samples. (A) Total proteins. (B) Low-abundance proteins. This figure extends the data presented in Fig. 2 to the group by illustrating total and low-abundance protein comparisons between the depleted and undepleted samples, combining all individual, again emphasizing the utility of depleting high-abundance plasma proteins to increase detection sensitivity and thus the number of proteins identified.

3.3 Comparative analysis of different cyst samples

The five cysts from which the kidney fluid samples were obtained were variable in diameter, appearance, protein concentration, and protein composition. Table 1 lists the cysts in the order of random assignment and lists the origin and characteristics of each cyst. Cyst 1 had an unusually low protein concentration of 1.58 mg/mL and cyst 2 had an unusually high concentration of 41.36 mg/mL. In comparison, plasma protein concentration is typically 39–50 mg/mL and glomerular filtrate protein ranges from 0.06 to 0.11 mg/mL (<http://www.lib.mcg.edu/edu/eshuphysio/program/section7/7ch04/7ch04p10.htm>). Previously, PKD renal

cyst fluid protein concentration was shown to range from 7 to 28 mg/mL [28]. CF01 fluid from cyst 1 had other unusual characteristics that included a brown, cloudy appearance and an abundance of mucin and ribosomal isoforms. One possible explanation for these unusual characteristics is that cyst 1 may have contained an adenoma [29].

Aside from the described differences in cyst 1, the relatively large size of cyst 5, and the elevated protein concentration in cyst 2, the cysts were similar in patient origin, color, fluid consistency, fluid volume, and protein concentration. Because PKD cysts typically range from barely visible to 500 mm, our sample cysts easily fall into that range (<http://radiology.uhc.edu/eAtlas/GU/1567.htm>).

3.4 Gene ontology annotation

The IPI numbers of all identified proteins common to at least three out of five cysts were entered into the Lewis-Sigler GO Term Finder. As such, the focus of this discussion will be on the 150 proteins common to at least 3/5 cysts. The IPI numbers corresponding to these 150 proteins were submitted to the GO Term Finder that recognized all but a few. In the individual ontological categories or “aspects”, some IPI numbers were recognized but were not annotated relative to any aspect in the database.

A review of unannotated IPI numbers yielded protein fragments, complement isoforms, and Ig fragments. In addition, due to the redundancy in the reporting of sub-categories by the web tool and our desire to visually simplify the histograms, some categories have been combined or nonspecific parent terms eliminated. This editing was done by consulting AmiGO (the official tool for searching and browsing the Gene Ontology database) to insure correct

Table 2. Human renal cyst fluid proteins common to at least three out of five renal cysts studied

Protein IPI number	Common name of protein	TPP confidence	Number of peptides
IPI00019943	Afamin precursor (vitamin E binding protein)	1.0000	6
IPI00550991	α -1-Antichymotrypsin, ACT, cell growth-inhibiting gene 24/25 protein	1.0000	15
IPI00847635	α -1-Antichymotrypsin; AACT	1.0000	15
IPI00553177	α -1-Antitrypsin, α -1 protease inhibitor, α -1-antiproteinase	1.0000	24
IPI00745089	α -1-B glycoprotein	1.0000	9
IPI00022895	α -1-B glycoprotein	1.0000	9
IPI00022426	α -1-Microglobulin/bikunin precursor	1.0000	11
IPI00029863	α -2-Antiplasmin precursor	1.0000	3
IPI00166729	α -2-Glycoprotein 1, zinc	1.0000	16
IPI00022431	α -2-HS-glycoprotein precursor	1.0000	6
IPI00032220	Angiotensinogen precursor	1.0000	4
IPI00784817	Anti-RhD monoclonal T125 γ 1 heavy chain precursor	0.9169	13
IPI00032179	Antithrombin III variant	1.0000	15
IPI00304273	Apolipoprotein A-IV precursor	1.0000	19
IPI00847179	Apolipoprotein A-IV precursor	1.0000	19
IPI00790930	BCL2-like 14 (apoptosis facilitator)	0.9882	1
IPI00298828	β -2-Glycoprotein 1 precursor	1.0000	9
IPI00796379	β -2-Microglobulin	1.0000	2
IPI00004656	β -2-Microglobulin	1.0000	2
IPI00852577	C1 segment protein (fragment)	1.0000	3
IPI00856060	C1 segment protein (fragment)	1.0000	3
IPI00795633	Clusterin	1.0000	2
IPI00400826	Clusterin isoform 1	1.0000	2
IPI00291262	Clusterin precursor	1.0000	2
IPI00784409	Coagulation factor II (thrombin)	0.9999	1
IPI00382606	Coagulation factor VII (serum prothrombin conversion accelerator)	0.9201	14
IPI00783987	Complement C3 precursor	1.0000	7
IPI00032258	Complement C4-A precursor	0.9999	1
IPI00654875	Complement C4-B precursor	0.9999	1
IPI00164623	Complement component 3	1.0000	7
IPI00643525	Complement component 4A	0.9999	1
IPI00019591	Complement factor B precursor (fragment), isoform 1	0.9999	2
IPI00165972	Complement factor D preproprotein	1.0000	2
IPI00515041	Complement factor H	0.9999	1
IPI00029739	Complement factor H precursor, isoform 1	0.9999	1
IPI00167093	Complement factor H-related 1	0.9993	1
IPI00011264	Complement factor H-related protein 1 precursor	0.9993	1
IPI00006154	Complement factor H-related protein 2 precursor, isoform long	0.9993	1
IPI00218949	Complement factor H-related protein 2 precursor, isoform short	0.9993	1
IPI00027482	Corticosteroid-binding globulin precursor	1.0000	4
IPI00032293	Cystatin-C precursor	1.0000	2
IPI00029717	Fibrinogen α -chain	1.0000	2
IPI00021885	Fibrinogen α -chain	1.0000	2
IPI00641047	Gelsolin	1.0000	6
IPI00796316	Gelsolin	1.0000	2
IPI00377087	Gelsolin	1.0000	2
IPI00026314	Gelsolin precursor, isoform 1	1.0000	6
IPI00646773	Gelsolin precursor, isoform 2	1.0000	6
IPI00022488	Hemopexin precursor	1.0000	22
IPI00022371	Histidine-rich glycoprotein precursor	1.0000	2
IPI00032328	HMW of kininogen-1 precursor, isoform	1.0000	4
IPI00430808	Ig- κ chain C region.	1.0000	3
IPI00387025	Ig- κ chain V-I region DEE	0.9974	2
IPI00550731	Ig- κ chain V-II region RPMI 6410 precursor	1.0000	3
IPI00386133	Ig- κ chain V-IV region B17 precursor	0.9661	5

Table 2. Continued

Protein IPI number	Common name of protein	TPP confidence	Number of peptides
IPI00386132	Ig- κ chain V–IV region JI precursor	0.9661	5
IPI00387120	Ig- κ chain V–IV region Len	0.9661	5
IPI00026197	Ig- κ chain V–IV region precursor, similar to	0.9661	5
IPI00784822	Ig-like (IGHV4–31 protein)	0.9169	13
IPI00784810	Ig-like (IGHV4–31 protein)	0.9169	13
IPI00440577	Ig-like (IGKV2–24 protein)	1.0000	3
IPI00829640	Ig-like (IGL@ protein)	1.0000	3
IPI00719373	Ig-like (IGL@ protein.)	1.0000	3
IPI00745660	Ig-like (IGL@protein)	1.0000	3
IPI00784828	Ig-like (putative uncharacterized protein DKFZp686C11235)	0.9169	13
IPI00784842	Ig-like (putative uncharacterized protein DKFZp686G11190)	0.9169	13
IPI00784865	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784985	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00430820	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784773	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00807459	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784661	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784589	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784711	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00785164	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784983	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784713	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784627	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00785079	Ig-like (putative uncharacterized protein)	1.0000	3
IPI00784894	Ig-like (putative uncharacterized protein)	1.0000	11
IPI00784807	Ig-like (putative uncharacterized protein)	1.0000	11
IPI00785200	Ig-like (putative uncharacterized protein)	0.9028	3
IPI00744561	Ig heavy constant α 1	1.0000	3
IPI00423466	Ig heavy constant γ 1	0.9169	13
IPI00829944	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00815926	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00807531	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00645363	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00448938	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00448925	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00423463	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00384938	Ig heavy constant γ 1 (G1m marker)	0.9169	13
IPI00423464	Ig heavy constant γ 1 (G1m marker)	0.9003	12
IPI00399007	Ig heavy constant γ 2	1.0000	11
IPI00829940	Ig heavy constant γ 3 (G3m marker)	1.0000	11
IPI00829850	Ig heavy constant γ 3 (G3m marker)	1.0000	11
IPI00829716	Ig heavy constant gamma 3 (G3m marker)	1.0000	11
IPI00827754	Ig heavy constant γ 3 (G3m marker)	1.0000	11
IPI00472345	Ig heavy constant γ 3 (G3m marker)	1.0000	11
IPI00816681	Ig heavy constant mu	0.9169	13
IPI00816314	Ig heavy constant mu	0.9169	13
IPI00761159	Ig heavy constant mu	0.9169	13
IPI00472610	Ig heavy constant mu	0.9169	13
IPI00418153	Ig heavy constant mu	1.0000	11
IPI00168728	Ig heavy constant mu	1.0000	11
IPI00785084	Ig heavy variable 4–31	0.9169	13
IPI00845354	Ig- κ constant	1.0000	3
IPI00853045	Ig- κ constant	1.0000	3
IPI00761125	Ig- κ constant	1.0000	3
IPI00746963	Ig- κ constant	1.0000	3
IPI00430847	Ig- κ constant	1.0000	3

Table 2. Continued

Protein IPI number	Common name of protein	TPP confidence	Number of peptides
IPI00816118	Ig-κ constant	1.0000	3
IPI00784070	Ig-κ constant	1.0000	3
IPI00472961	Ig-κ constant	1.0000	3
IPI00827488	Ig-κ constant	1.0000	3
IPI00854806	Ig-κ variable 1–5	1.0000	3
IPI00478600	Ig-κ variable 1–5	1.0000	3
IPI00419424	Ig-κ variable 1–5	1.0000	3
IPI00294193	Inter-α (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein)	1.0000	3
IPI00790993	Inter-α-trypsin inhibitor	1.0000	3
IPI00218192	Inter-α-trypsin inhibitor heavy chain H4 precursor, isoform 2	1.0000	3
IPI00789376	Kininogen 1	1.0000	6
IPI00797833	Kininogen 1	1.0000	6
IPI00215894	Kininogen-1 precursor, isoform LMW	1.0000	6
IPI00022417	Leucine-rich α-2-glycoprotein precursor	1.0000	4
IPI00020986	Lumican precursor	1.0000	3
IPI00418163	MHC class III region complement	0.9977	1
IPI00022429	Orosomuroid 1, α-1-acid glycoprotein 1 precursor	1.0000	5
IPI00006114	Pigment epithelium-derived factor precursor	1.0000	3
IPI00022420	Plasma retinol-binding protein precursor	1.0000	2
IPI00513767	Prostaglandin D2 synthase 21 kDa (brain)	1.0000	3
IPI00013179	Prostaglandin-H2 D-isomerase precursor	1.0000	3
IPI00019568	Prothrombin precursor (fragment)	0.9999	1
IPI00480192	Retinol binding protein 4, plasma	1.0000	2
IPI00844536	Retinol binding protein 4, plasma	1.0000	2
IPI00816799	Rheumatoid factor D5 light chain (fragment).	0.9993	2
IPI00815938	Rheumatoid factor G9 light chain (fragment)	1.0000	3
IPI00426051	Rheumatoid factor RF-IP15 (fragment)	1.0000	5
IPI00022463	Serotransferrin precursor	1.0000	27
IPI00556459	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary)	1.0000	2
IPI00291866	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary)	1.0000	2
IPI00745872	Serum albumin (precursor)	1.0000	61
IPI00798430	Transferrin variant	1.0000	27
IPI00760855	Transmembrane protein 110	1.0000	3
IPI00646384	Transthyretin (prealbumin, amyloidosis type I)	1.0000	3
IPI00855916	Transthyretin (precursor), prealbumin, TBPA, TTR, ATTR	1.0000	3
IPI00022432	Transthyretin precursor, prealbumin	1.0000	3
IPI00555812	Vitamin D-binding protein precursor	1.0000	19
IPI00742696	Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin)	1.0000	19
IPI00298971	Vitronectin precursor	1.0000	2

Peptides and proteins were identified by SEQUEST and validated *via* the TPP *via* PeptideProphet and ProteinProphet. Only those identified with ≥90% confidence are listed, using IPI accession numbers common protein name.

attribution of subcategories and to respect the gene ontology dendrogram [30]. These annotated proteins grouped by subcategories are included in a graphical display in Figs. 4–6. Ontologies are explained fully on the Gene Ontology website: (<http://www.geneontology.org/GO.doc.shtml#ontologies>).

Figure 4 illustrates proteins categorized by the gene ontology Molecular Function aspect. A large number of identified cyst proteins function as protease inhibitors, spec-

ifically serine-type endopeptidase inhibitors. Serine-type endopeptidases include trypsin, thrombin, and most notably numerous coagulation-pathway enzymes. The identified inhibitors of interest include antichymotrypsin, angiotensinogen, antithrombin III, α-2-antiplasmin, and C1 inhibitor. Antigen binding is also a function of many of the proteins identified in the sample. A review of the antigen binding proteins shows that they are predominantly Ig heavy and

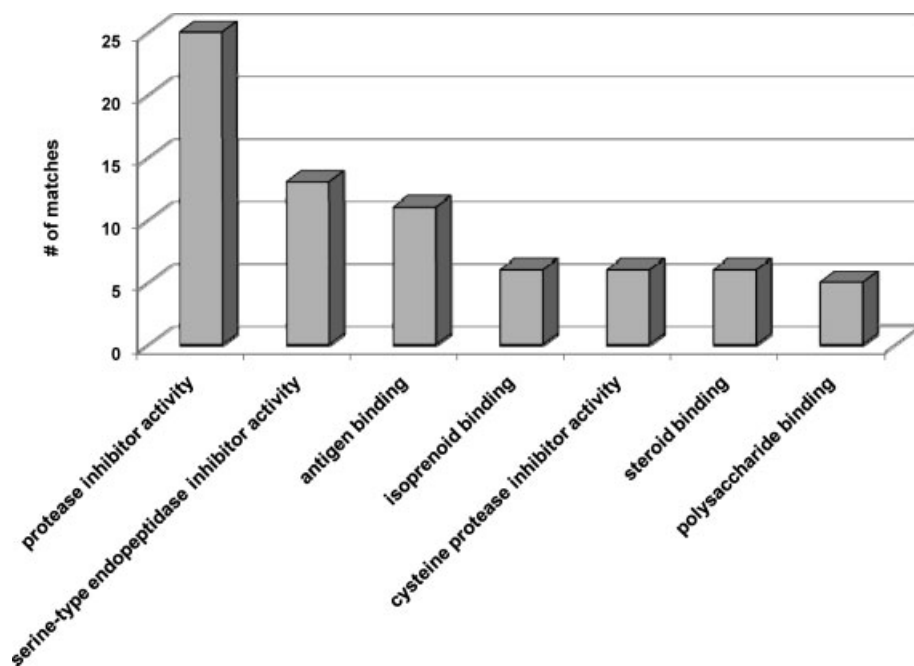


Figure 4. The results of the GO Term Finder query of the “Molecular Function” aspect using 150 proteins from renal cyst fluid common to at least three out of five samples analyzed. The ordinate value is the actual number of IPI numbers that match to each GO Term. (<http://www.geneontology.org/GO.function.guidelines.shtml>).

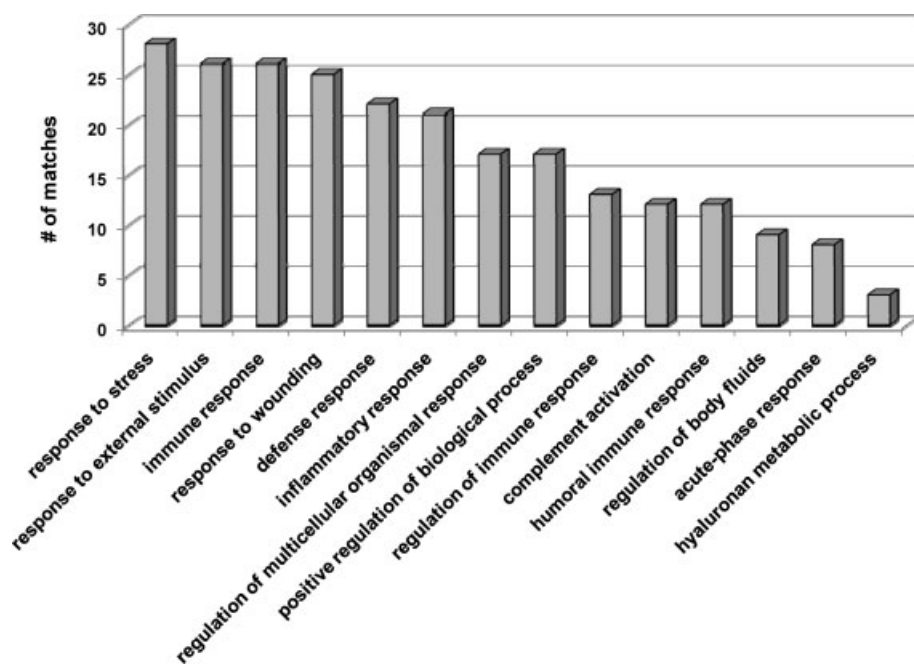


Figure 5. The results of the GO Term Finder query of the “Biological Process” aspect using 150 proteins from renal cyst fluid common to at least three out of five samples analyzed. The ordinate value is the actual number of IPI numbers that match to each GO Term. (<http://www.geneontology.org/GO.process.guidelines.shtml>).

κ -chains, as would be expected. Isoprenoid binding proteins appear in the sample as vitamin A binding proteins. It is important to note that vitamin A has a role in growth and differentiation [30] and its presence in the fluid may reflect such an effect on the expanding cyst.

Figure 5 illustrates the number of proteins categorized according to the Biological Process aspect. This category includes a high proportion of proteins that are involved in

responses to external stimulus, wounding, and pathogens. Defense response proteins restrict damage to an organism caused by an infection. In the present study, most of these proteins are likely to have been proteolyzed to active enzymes such as complement components. Complement activation proteins enable the direct killing of microbes, the disposal of immune complexes, and the regulation of other immune processes [31]. Regulation of body fluids is another

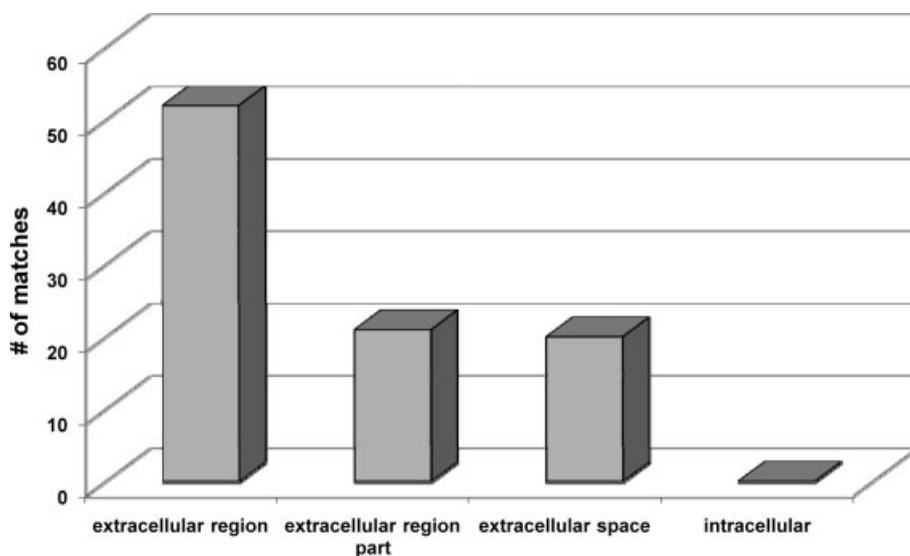


Figure 6. The results of the GO Term Finder query of the “Cellular Component” aspect using 150 proteins from renal cyst fluid common to at least three out of five samples analyzed. The ordinate value is the actual number of IPI numbers that match to each GO Term. According to the curators of the GO database, extracellular region is defined as “the space external to the outermost structure of a cell. For cells without external protective or external encapsulating structures this refers to space outside of the plasma membrane. This term is intended to annotate gene products that are not uniformly attached to the cell surface. For gene products from multicellular organisms which are *secreted* from a cell but retained within the organism (*i.e.*, released into the interstitial fluid or blood), consider the cellular component term ‘*extracellular space*.’” Extracellular region part includes “any constituent part of the extracellular region, the space external to the outermost structure of a cell” and is thus a more specific subset of extracellular region. Finally, intracellular is defined as the contents of the cell excluding the plasma membrane and any structures outside the plasma membrane. (<http://www.geneontology.org/GO.component.guidelines.shtml>).

category of interest. These proteins include antithrombin, β -2-glycoprotein, angiotensinogen, and complement inhibitors [30].

In the subcategories comprising the cellular component aspect, all identified and annotated proteins fell into the extracellular region category. A more specific designation suggests that many of these proteins are secreted into the extracellular space. This would be expected of cyst fluid, and emphasizes the importance of concomitantly analyzing the proteome of epithelial cells generating this extracellular fluid in future studies.

3.5 Notable proteins

Over 350 unique proteins were identified in PKD cyst fluid spanning all five samples studied. A significant fraction of these were recognized as Ig fragments, protein precursors, and isoforms. Nonetheless, many distinct protein identifications were made, representing an abundant source of protein molecules potentially involved in cyst maintenance, growth, and structural formation.

For instance, vitronectin is a protein of interest because it serves as a receptor for the integrin α -v- β 3, an adhesion, and angiogenesis molecule. A study by Bello-Reuss *et al.* demonstrated neovascularization in PKD cyst walls along with expression of α -v- β 3. Neovascularization may be necessary

for cyst growth and contribute to increased vascular permeability and fluid secretion [32].

A study by Woo [33] showed that, in addition to cyst enlargement and interstitial fibrosis, apoptosis is a pathological feature of PKD. Apoptotic cells are phagocytized within a few hours by neighboring cells or by phagocytes in a process involving the vitronectin receptor or the phosphatidyserine receptor. In human PKD, where nephron loss is slow, apoptosis was nevertheless detected before the onset of uremia.

The sulfated glycoprotein clusterin (Apo J) has been detected in significant quantity in hepatic cyst fluid [34] and is a potential biomarker of PKD [35, 36]. Urinary clusterin has been found to be elevated in (cy/+) rat model of ADPKD rats with progressive PKD [37]. Clusterin also interacts with complement components and vitronectin [38], where they bind to the membrane attack complex (MAC) and prevent cytolysis. Despite this apparent inhibitory effect, patients in that study diagnosed with lupus nephritis were noted to have more renal pathology with elevated serum vitronectin and clusterin. It is interesting that many components of the complement cascade were identified in our cyst fluid samples, but it is unknown if the complement system is involved in the pathology of PKD [38].

The renal cyst fluid contains a large number of proteins from the SERPIN (serine-protease inhibitor) family. This protein family inhibits the proteases that function as coagu-

lation factors, complement system components, and digestive enzymes. Although there is no described mechanism of how this class may contribute to PKD, we cannot ignore the consistent identification of multiple, unique SERPINS in the various human cyst fluid samples studied here. Many of these proteins may be leaking into cysts from a plasma source, as about 10% of plasma proteins are SERPINS [39]. Nevertheless, these proteins are excellent candidates for the further experimental investigation.

β -2-Glycoprotein has been associated with renal disease, but more as a marker than as an etiologic agent. Other proteins in our study, such as α -1-microglobulin, retinol binding protein, and β -2-microglobulin have also been identified as protein markers of tubular malfunction. These proteins are low molecular weight markers that are freely filtered and normally are reabsorbed by renal tubule cells [40]. These proteins may have a pathological role in PKD, or, alternatively, could be markers for disease severity or cyst stage.

Fetuin-A (α -2-HS glycoprotein) has been correlated with epithelial cell toxicity in the nephron and has been detected in urinary exosomes. Zhou *et al.* [41] hypothesize that diseased and sloughing proximal tubular cells generate fetuin-A. They note that immature rat renal cells synthesize fetuin-A, and this protein likely plays a role in cell differentiation and tissue transformation during the initial histogenesis [42]. The identification of fetuin-A in our cyst fluid samples strongly suggests a degree of dedifferentiation in PKD renal epithelial cells [41].

Hemopexin is secreted into the plasma in response to inflammation (acute phase reactant) and it binds heme with high affinity [43]. As mentioned above, this protein has been identified previously in renal cyst fluid studies [12]. Hemopexin has been shown to cause proteinuria after direct renal infusion. This pathology is due to a protease action and results in microscopic changes in glomerular histology [44]. It is unknown if this contributes to the pathological damage in observed in PKD.

A preponderance of Ig and complement molecules are identified in the cyst fluid. It is not clear if these Ig proteins are simply diffusing into the cyst from plasma or if they are locally produced by B-cells. An intriguing explanation is that there may be an immunological component to this disease. Perhaps those cells expressing altered forms of polycystin or other unrecognized protein are inducing an immunologic response. With variable phenotypic expression and progression to renal failure in ADPKD, it would seem that an immunologic mechanism could be at play here [1]. A recent study shows high levels of complement component 3 in models of ARPKD and suggests abnormal activation of complement is a key component in cystic disease progression [45].

Several proteins of interest in PKD, such as polycystin 1 and 2, fibrocystin, TGF, and EGF are absent from our samples. The polycystin protein complex would not be expected to be detected by mass-spectrometry in the end-stage cyst fluid *per se*. An epithelial cell or exosomal proteomic study

would be expected to yield polycystin and other intracellular/membrane-bound proteins [46]. It is also noted that TGF and EGF are absent from our sample. In a study by Quigley *et al.* [47], it was found that filtrated EGF is bound to albumin with a free fraction of $0.31 \pm 0.04\%$. These proteins may part of the albumin bound peptidome in our sample and therefore depleted. Future experiments to find low-abundance proteins/polypeptides attached to albumin will be conducted in our laboratory.

4 Concluding remarks

Our analyses of human cyst fluid identified a large number distinct proteins along with hundreds of protein precursors and isoforms. Depletion of common plasma proteins yielded additional protein identifications. A query of the gene ontology databank demonstrated that our proteins are located in the extracellular region; the main protein functions include serine protease inhibition and antigen binding; the protein processes include organism defense, reaction to stimulus, and regulation of body fluids. Proteins of specific interest include vitronectin, clusterin, SERPIN family proteins, hemopexin, fetuin-A, and complement components. These identified proteins may offer mechanistic explanations for cyst development and maintenance, serve as markers for diagnosis and monitoring, or serve as potential targets for therapeutic intervention of PKD. Future studies should include quantitative comparisons of hepatic and renal cyst fluid from rodent models, comparison of human cyst samples to these rodent models, and a survey of the albuminome [48, 49] depleted from our cyst fluid samples.

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