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A prospective, proteomics study identified potential biomarkers of encapsulating peritoneal sclerosis in peritoneal effluent.

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1

Abstract:

Encapsulating peritoneal sclerosis (EPS) is a potentially devastating complication of peritoneal dialysis (PD). Diagnosis is often delayed due to the lack of effective and accurate diagnostic tools. We therefore examined peritoneal effluent for potential biomarkers that could predict or confirm the diagnosis of EPS and would be valuable in stratifying at-risk patients, and driving appropriate interventions. Using prospectively collected samples from the Global Fluid Study and a cohort of Greek PD patients, we utilized 2D SDSPAGE/ MS and iTRAQ to identify changes in the peritoneal effluent proteome from patients diagnosed with EPS and controls matched for treatment exposure. We employed a combinatorial peptide ligand library to compress the dynamic range of protein concentrations, to aid identification of low-abundance proteins. In patients with stable membrane function, fibrinogen γ-chain and heparan sulphate proteoglycan core protein progressively increased over time on PD. In patients who developed EPS, collagen-α1(I), γ-actin and Complement factors B and I were elevated up to five years prior to diagnosis. Orosomucoid-1 and a2-HS-glycoprotein chain-B were elevated about one year before diagnosis, while apolipoprotein A-IV and α1-antitrypsin were decreased compared to controls. Dynamic range compression resulted in an increased number of proteins detected with improved resolution of protein spots, compared to the full fluid proteome. Intelectin-1, dermatopontin, gelsolin and retinol binding protein-4 were elevated in proteome-mined samples from patients with EPS compared to patients that had just commenced peritoneal dialysis. Thus, prospective analysis of peritoneal effluent uncovered proteins indicative of inflammatory and pro-fibrotic injury worthy of further evaluation as diagnostic/prognostic markers.

Keywords: peritoneal dialysis, encapsulating peritoneal sclerosis, biomarkers, proteomics

2

Introduction:

Encapsulating peritoneal sclerosis (EPS) is a rare complication of peritoneal dialysis (PD) resulting in significant morbidity and mortality[1]. Overall reported life-time risk ranges from 0.54%[2] to 3.3%[3]. Incidence increases with the duration of PD treatment [4-6], ranging from 0.7%[4] to 8.1%[5] at 5 years and from 2.1%[4] to 19.4%[6] at 8 years. The development of EPS has been associated with numerous factors including glucose load, rapid solute transport status and intraperitoneal inflammation, requirement for icodextrin use, ultrafiltration failure associated with reduced free water transport, modality change, young age and genetic predisposition [7-14].

The pathogenesis of EPS remains poorly defined but multiple "hits" appear to be required in patients predisposed to peritoneal injury by uremia and dialysis solution exposure.[15] Although episodic infection, epithelial-to-mesenchymal transition (EMT)[16] and the involvement of uncontrolled inflammatory cascades (TGF-beta, VEGF etc.)[17-20] may be contributory factors, to date no single definitive pathway has been identified.

Clinical signs of EPS include persistent or recurrent bowel dysfunction and nutritional impairment[1]. Diagnosis is confirmed at laparotomy or on PET/CT imaging[21], but the rarity of the disorder, variable presentation and lack of screening tools frequently lead to delayed diagnosis. Peritoneal dialysis effluent (PDE) biomarkers that could predict the onset or confirm the diagnosis of EPS would allow the development of prognostic tools and, potentially, the identification of future therapeutic targets. Proteomics technologies provide us with powerful tools for an in-depth exploration of the PDE proteome, yet relevant studies have been few and to date preliminary [22-38].

In the present study, we have used an optimised proteomics approach to detect prospective changes in the PDE proteome of clinically well-described patients from the Global Fluid Study (GFS) cohort who subsequently developed EPS. Controls were patients from the same cohort with stable membrane function who did not subsequently develop EPS. EPS and stable patients were matched for time on treatment or time to developing EPS/stopping PD. The aim of this study was to identify

3

proteins in the drained PD effluent whose measurement might predict the onset of EPS. Further

analysis, using a cohort of Greek PD patients, utilized methods to allow detection of lower abundance

proteins by using a combinatorial peptide ligand library (CPLL). Based on these experiments, our data

identifies proteins whose expression changes in EPS patients and in some cases are expressed in

PDE significantly in advance of clinical diagnosis. These data support growing evidence that EPS is

preceded by inflammatory and fibrotic peritoneal injury and would seem worthy of further exploration

as candidate markers of EPS diagnosis and monitoring.

4

Results:

Determination of protein changes with time on PD in patients with stable peritoneal membrane function

To ascertain the time-course of changes in the levels of proteins in PDE, samples from 5 patients with at least 10 sequentially collected effluent samples spanning a period of >4 years on PD, were analysed by 2D PAGE. Over 800 protein spots were consistently detectable in all samples when analysed by PDQuest. The majority of these proteins were relatively constant in expression providing confidence of the validity and fidelity of the methodology selected (Figure 1). PDQuest identified approximately 25 spots that either increased (x2) or were reduced (x2) in intensity in a progressive manner with dialysis duration. Of these, however, only 2 spots were consistently changed in greater than 4 out of the 5 patient samples evaluated. Mass spectrometry identified these proteins as fibrinogen γ-chain (Figure 2a) and Heparan-Sulphate Proteoglycan (HSPG) (Figure 2b). Based on spot intensities, fibrinogen γ-chain began to be elevated after approximately 30% of total dialysis duration before plateauing while HSPG levels showed a progressive increase over the entire duration of PD treatment.

Determination of protein changes in patients that developed EPS

A. 2D SDS PAGE MS proteomics

To determine which proteins were changing in patients who were ultimately diagnosed with EPS, 6 UK patients from the GFS, who had EPS diagnosis confirmed by CT scan, and who had ≥4 sequential samples prior to this diagnosis were selected. Each of these patients was matched to a patient from the same centre according to the following criteria: equal exposure to PD treatment, comparable sampling points from the time of initiation of PD, stable membrane function and lack of any evidence indicative of EPS (see Supplemental Table 1 for time point matching details). The Dodeca gel system that was used for the study runs a maximum of 12 gels, so 6 EPS gels and 6

5

matched control sample gels was the maximum comparison that could be performed in a single experiment. Twenty four spots were identified in all the EPS patients as being differentially altered over time compared to the matched patients control samples. Of these, 2 spots were identified as being altered consistently in all of the 6 EPS patients in comparison to the control patients. Collagen-α1(I) became elevated in EPS patients (Figure 3a) typically after 30% of total dialysis duration, while γ-actin (Figure 3b) was frequently elevated very early (124-1043 days) and remained elevated throughout the entire duration of PD (maximum duration was 2868 days) until EPS was diagnosed clinically.   In 5 out of the 6 EPS patients, changes in spots subsequently identified as complement factors B and factor I were seen (Figure 4). The complement factor B spot was either not present or barely detectable in matched patients with stable membrane function, but was elevated (in some patients up to 6 years before EPS was diagnosed) and remained elevated (above matched control levels) in those patients who were subsequently diagnosed with EPS. The Complement factor I spot was present in both patient groups and decreased slightly with duration of PD. It was, however, at consistently higher density at each time point in those who subsequently were diagnosed with EPS. α1-antitrypsin and apolipoprotein A-IV spots appeared at noticeably lower intensity in 3 out of 6 EPS patients.

B. Isobariq tagging for relative and absolute quantification (iTRAQ) proteomics.

2D SDS-PAGE is a labour-intensive approach, with analysis limited by the accuracy of spot mapping software that subsequently needs a high degree of manual mapping and is subject to data losses. To address these issues we optimised a mass spectrometry based iTRAQ protocol for use with PDE samples and repeated the comparisons in one EPS and matched control sample pair. An 8 iTRAQ approach was used allowing comparison of 4 time points from each patient. A limitation of iTRAQ is the low rate of protein detection, however 72 proteins had sufficient sequence recognition to allow for robust protein identification (Table 1). Of these, by performing ratio analysis of the isobariq tag from each patient at each time point it was possible to identify 4 consistent trends (Figure 5).

6

Orosomucoid-1 and ·2-HS-glycoprotein chain B were both elevated in the EPS patient approximately 12 months prior to diagnosis, while Apolipoprotein A-IV was always lower in EPS. There was also a late drop in serotransferrin levels at EPS onset.

Protein mining.

From both SDS-PAGE and iTRAQ it was evident that >95% of the total protein load detected was highly abundant serum proteins such as albumin and IgG (Figure 6A). This meant that <5% of the proteins being analysed were ones of potential interest. To address this, a protein-mining approach was used employing the Bio-Rad® ProteoMiner™ enriching system to reduce the dynamic range of abundant protein concentrations, while maintaining the relative levels of less abundant proteins. In comparison to the full fluid proteome where approximately 800 spots were identified (many of which were related) (Figure 6A), post ProteoMiner™ analysis the same protein concentration yielded >2000 discrete protein spots that were all well resolved (Figure 6B).

Determination of lower abundance proteins changing in patients that developed EPS using 2D SDS-PAGE/MS proteomics on samples subjected to ProteoMiner.

Protein mining required a starting protein mass of approximately 15mg, to produce 60μg of protein for proteomic analysis. In the GFS cohort, sufficient individual patient PDE was not available for this type of analysis. However, as part of a collaborative study with the University of Patras, Greece, we were able to obtain significant PDE volumes from 3 patients with suspected EPS. These samples were compared to 3 prevalent patients with stable membrane function who had been on PD for >5 years and with 3 incident patients from the same centre. All 9 samples were subjected to ProteoMiner™ analysis, fractionated by 2D-SDS PAGE and compared using PDQuest™. From this analysis, three spots were detected that were elevated only in the PDE of patients with EPS. Intelectin-1 (Figure 7a) was barely detectable in patients just starting PD or in patients who had been on PD for >5 years. However, in patients with suspected EPS a strong protein spot was visible.

7

Likewise, dermatopontin (Figure 7b) was also strongly expressed in EPS patients, but was only mildly

elevated in 1 of the 3 prevalent patients and not at all in patients starting PD. Retinol binding protein-4

(RBP-4) was also identified as increasing in EPS patients (not shown).   There were numerous

additional spots seen but we were subsequently unable to identify them in our MS analysis.

Patient pooling to facilitate protein mining and ITRAQ on the GFS cohort

To combine the benefit of protein-mining, the sequential sampling of patients in the GFS, the

power of the iTRAQ approach and provide sufficient protein for meaningful analysis, we combined

GFS effluent samples from several patients into 4 groups based on time on dialysis for both stable PD

patients and for those developing EPS. Samples were pooled in to Group 1; within 6 months of EPS

or stopping dialysis, Group 2; 6-12 months prior to stopping dialysis, Group 3; 12-24 months prior to

stopping dialysis and Group 4; >24 months before cessation of dialysis. Each pool of fluid (~18mg of

protein, see supplemental table 2 for pool composition detail) was protein-mined using proteominer,

iTRAQ-labelled and analysed by MS as previously described (Figure 8).

Analysis identified >2,000 detectable proteins and sequence recognition allowed robust

identification of 136 of these proteins. Hierarchical cluster analysis showed a clear demarcation of

proteins found between those who develop EPS and the control-matched patient samples (Figure 9a).

Heat map analysis (Figure 9b) demonstrated higher levels of many of these proteins in the EPS

effluent samples across all time points analysed.   Subsequent analysis of the ratios of relative

changes in individual proteins between EPS and non-EPS patients with time was disappointing, in that

consistent progressive changes with time for individual proteins were limited compared to numerous

ratio shifts at individual time points.   Nevertheless, the data confirmed the previously observed

increases in intelectin-1 expression and a small reduction in Apolipoprotein with both A-II & A-chains

being reduced in samples from patients subsequently developing EPS.   Interestingly, despite

numerous changes being observed at each time point only two additional proteins appeared to change

progressively. Multiple Haemoglobin subunits were dramatically increased within the last 12 months in

EPS patients despite no macroscopic evidence of blood in the PDE samples, whilst Gelsolin showed a progressive accumulation with time (Figure 10).

Discussion:

The combination of uraemic milieu and chronic exposure to PD solutions induce the development of structural changes in the peritoneum[39-40]. There is significant interest as to whether these histological changes can be correlated with or predicted by changes in effluent proteins (irrespective of source) and whether the levels of these proteins are altered with duration of PD. Such protein changes, if consistent, might provide easily detectable effluent biomarkers of distinct peritoneal pathology or have routine prognostic value. In the present study we chose to screen for effluent proteins in defined groups of PD patients using a broad-based proteomics approach. We used prospectively collected PDE samples from the uniquely characterized patients in the GFS cohort to examine if specific proteins were identifiable and ask if any of these were specifically changed in patients who subsequently developed EPS. The 16 proteins identified across all proteomics approaches, (which employed up to 10 patients that developed EPS, matched with 18 with stable function) are summarized in table 2. Given that the function of some of these proteins is already known, we can hypothesise as to their potential role in EPS.

Fibrin deposition is a feature of peritoneal fibrosis and EPS pathology[15]. Fibrin is derived from fibrinogen and both are involved in inflammatory processes. In PDE fibrinogen γ-chain increases during peritonitis and declines after successful treatment[28]. Our results indicate that the amount of fibrinogen γ-chain in PDE begins to increase after 6-18 months on PD and plateaus thereafter. As major histopathological changes in the dialysed peritoneum begin to manifest fully after ~2 years on PD[39], the pattern of fibrinogen γ-chain elevation might reflect the evolution of processes that contribute to the establishment of peritoneal fibrosis. Fibrinogen γ-chain may thus become elevated as fibrogenesis is initiated and plateau when fibrosis becomes established. Peritoneal fibrosis is also characterised by increased turnover of extracellular matrix (ECM). This process might be reflected by

9

the progressive increases of HSPGs[41] in PDE, as they are key components of basement membranes.

Using 2D SDS-PAGE/MS, we observed increases in the concentrations of collagen-α1(I) and γ-actin in the PDE of 6 patients subsequently diagnosed with EPS. These changes were not seen in the matched control patients. Elevation of collagen-α1(I) may reflect increased ECM turnover and the deposition of type-I collagen in the peritoneal tissues[42]. Expression of collagen-α1(I) mRNA is up-regulated in EPS patient peritoneum, while its accumulation in the sub-mesothelial compact zone and EPS-derived tissue has been demonstrated by immunostaining[43-44]. Remodelling of γ-actin cytoskeleton is a feature of EMT[45]. Early elevation of γ-actin in PDE might be the result of its release from damaged peritoneal tissues[46]. Interestingly, the changes in γ-actin are consistent with the observed increases seen in gelsolin in the pooled EPS cohort samples. Gelsolin is an actin-binding protein that is a key regulator of actin filament assembly and disassembly and one of the most potent members of the actin-severing gelsolin/villin superfamily. Its elevation might help to explain the increased γ-actin levels observed in PDE.

In contrast, α1-antitrypsin and apolipoprotein A-IV were both at noticeably lower levels in patients diagnosed with EPS. α1-antitrypsin (1-PI) is an inhibitor of ECM proteases including plasmin and suppresses the inflammatory response in PDE by inhibiting PAF biosynthesis. Blocking of 1-PI by oxidants during inflammatory process allows for increased production of PAF and unrestrained synthesis of inflammatory mediators[47]. Apolipoprotein A-IV has anti-inflammatory[48] properties. Therefore, lower levels of 1-PI and apo A-IV may indicate an active inflammatory process which might contribute to EPS development[49]. The latter is further supported by the observed increases in Complement factor expression[43]. Complement Factor I and factor B were consistently detected prior to (years before) and remained elevated in all patients who subsequently developed EPS. Upon activation of the alternative pathway, Factor B is cleaved by complement factor D yielding the catalytic subunit Bb. The active subunit Bb associates with C3b to form the alternative pathway C3-convertase, driving subsequent complement activation and inflammation. Factor I (also known as

10

C3b/C4b inactivator) is a key regulator of downstream complement activity and its elevated levels are a further indicator of ongoing inflammatory activation in EPS patients.

Using iTRAQ analysis we demonstrated alterations in the effluent concentrations of acute phase proteins (APP). Orosomucoid-1 and a2-HS-glycoprotein chain B were both elevated in EPS patients >12 months prior to diagnosis whilst Apolipoprotein A-IV was always low in EPS patients. Orosomucoid-1 is a major APP. Its concentration increases several-fold in response to inflammation, infection or systemic tissue injury[50-51]. In PD patients, its dialysate levels increase during peritonitis[52]. Alpha2-Heremans–Schmid glycoprotein (AHSG), or fetuin-A, has been suggested as a negative APP of inflammation[53] but as a positive APP of injury[54] and as an antagonist of TGF-β[55]. The observed increase of these two molecules in the PDE, months before the onset of overt EPS, might underline a potential prognostic value. Serotransferrin is the major iron transporter and is down-regulated in inflammatory conditions[56-57]. The observed late drop of serotransferrin levels might thus also reflect an increased inflammatory event that is critical in the establishment of full-blown EPS symptoms and pathology.

Complex biological samples are characterised by a high dynamic range of protein concentrations[58], posing a major challenge in proteomic analyses[59] as less abundant components are far more likely to be useful as biomarkers. Combinatorial peptide ligand libraries (CPLL) can compress the dynamic range by altering the proportion of abundant versus non-abundant proteins[60]. This approach was preferred over depletion protocols, where proteins of potential interest may also be removed non-specifically by sticking to depleted proteins such as albumin. Recovery of <1% of treated protein, increased the number and improved resolution of spots, indicating that proteome-mining was successful and increasing the sensitivity of 2D SDS-PAGE. Variability in spot intensities indicates that quantitative information was not lost during the mining process. Comparisons between gels from CPLL-mined samples identified that intelectin-1, dermatopontin and retinol binding protein-4 appeared to be elevated in PD patients diagnosed with EPS compared to matched controls or those just starting on PD. Intelectin-1 is an adipocytokine involved in intestinal host defences[61].

11

Dermatopontin is an ECM protein that regulates the function of TGF-β by interacting with the proteoglycan decorin[62]. Its presence in PDE might thus indicate increased turnover and accumulation of ECM in the peritoneum[63], being consistent with changes in Collagen 1 and HSPG.

Overall the primary aim of this study was to identify early biomarkers for subsequent validation that would predict later risk of EPS. This has been successful in identifying changes in Collagen $\alpha1(1)$, γ-Actin, complement I & B as well as anti-trypsin up to 3 to 5 years before EPS onset, with Gelsolin, Apo AII, Apo-AIV & haemoglobin β changing at least 2 years before onset, although caution should be exercised with these time frames due to the large variation in time on PD between the matched populations. The study also identified late markers such as serotransferrin, Orosomucoid-1, Alpha-2-HS-glycoprotein chain B and intellectin-1 which may have diagnostic value when combined with symptoms. One could further hypothesise that this data supports the multiple hit theory of EPS with early, mid and late protein changes in that there may be a time-point where a "second hit" drives the process into the EPS pathway post an initial hit priming the system (eg. reflected by changes in Collagen I and Actin). From that time on, the inflammatory and other processes that lead to the development of EPS may need 6-12 months to fully develop into a recognisable clinical syndrome. Its thus likely, and reflected here, that biomarkers would fall into the same 2 categories.

While of interest, the robustness of our findings is significantly limited by the low number of patients available for suitable stratification and the limited numbers that can be compared in these proteomic approaches. The GFS is one of the world's largest PD cohort studies, yet has limited EPS-to-control matching options, resulting in some differences in descriptive data to remain apparent. As with all previous studies in this area, it is possible that differences in measured and unmeasured confounders could be biasing the results. Further, the selected proteomics approach was not able to determine the source of the proteins identified in this analysis and a possible spill-over effect from plasma cannot be excluded which needs to be addressed in ongoing investigations.

Previous studies have suggested that a number of proteins, primarily inflammatory biomarkers (IL-6, TNF-α, MCP-1, CCL-15, PAI-1), were slightly increased in the PDE of patients who

subsequently developed EPS[10,49,64-65], but were not identified by our proteomics approach. Previous studies used a targeted ELISA based approach, which has high sensitivity and subsequently resolution. Proteins identifiable with ELISA may subsequently fall below the detection limit of the proteomics approaches described, especially where protein-mining was not undertaken. Further, the proteomic technique may be unable to see the protein in the matrix used. Protein spots may not resolve, while high-matching criteria (4 peptides minimum) may have disadvantaged detection of smaller proteins. Alternatively, proteins may have not flown in the MS or not label with an isobariq tag. Finally, the small number of patients, the frequency of the change (i.e. spot changes in >75% of comparisons) or its magnitude could impede identification. Multiplexing approaches are more sensitive and quantitative, and could have been used here. However the investigator must select the list of proteins to measure, which precludes discovery of new protein involvement. Further these approaches demand high sample number and would not add power to the results described due to the very small number of EPS samples in the GFS cohort.

In contrast, the strengths of this study lie in its prospective nature, its unbiased nature and multiple proteomic approaches. Prospective analysis has resulted in identifying changes that occur consistently over time (and prior to disease onset in many cases), are robust across multiple comparisons and differentiate between EPS and control patients. The discovery-based approach, has allowed the analysis to be undertaken without prejudice for already known proteins and pathways, thus reducing protein selection bias and potentially allowing the discovery of novel markers of peritoneal fibrosis and EPS.

Collectively, the study results demonstrate that progressive and consistent changes in th e PDE proteome can be identified in PDE samples derived from patients who subsequently developed EPS. The majority of the proteins identified in EPS patients are consistent with their potential involvement in inflammation, the acute phase response, fibrogenenesis and ECM turnover (Table 2). This novel data supports the need for further investigation using classical measurement techniques. Future studies should focus on the quantitative investigation of the proteins identified here and in other

studies, in large, multicenter, well-stratified PD patient cohorts to provide robust validation of the use of

these proteins as potential predictive and diagnostic tools, either individually or as part of a fingerprint.

14

Methods

(Comprehensive description of methods used provided in supplementary material)

Patient Cohorts, Matching and Sample Collection

Patients were drawn from the Global Fluid Study (GFS)[66] and a smaller single-centre cohort from the University of Patras, Greece. The GFS cohort provided prospectively collected effluent samples from patients with confirmed diagnosis of EPS and control patients primarily matched for treatment exposure (time on PD or time to EPS/stopping PD) and centre (Supplemental tables 1&2). The Greek cohort provided single, large volume samples from nine selected patients, needed for the optimization of Proteominer™ for use with PDE. The study was conducted adhering to the tenets of the Declaration of Helsinki and informed consent was obtained from all patients. Ethical approval was obtained from the Multi-Centre Research Ethics Committee for Wales covering the UK, whilst local country ethics were obtained for other contributing countries. The clinical and demographic characteristics of participating patients from both cohorts are summarised in Table 3a and 3b, respectively.

Sample preparation

Total protein was precipitated using acetone/100%TCA, and resolubilised in buffer. 60μg of protein were loaded on pH 4-7 immobilised pH gradient (IPG) strips (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.).

Two-dimensional electrophoresis

Isoelectric focusing (IEF) was performed in a PROTEAN IEF system using a four-step protocol and subsequently, proteins were fractionated on a 20 x 23cm 10% polyacrylamide gel using a PROTEAN Plus Dodeca Cell system (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.).

Gel fixing and silver staining

15

Gels were fixed using 40% methanol and 10% acetic acid and silver-stained as described elsewhere.[67]

Gel Imaging

Gels were scanned on a Genetools densitometer (transmission) and PDQuest v8.0 (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.) was used for image analysis.

Proteome mining

Proteominer™ (Biorad, Laboratories Ltd., Hercules, CA, U.S.A.) was employed, using a 20µl of beads to 10 mg of protein ratio and a two-step elution protocol.[68]

iTRAQ labeling

Sample labeling with iTRAQ reagents was performed as described elsewhere.[69] Briefly, 100 µg of protein from each sample was reduced, alkylated and digested with trypsin, prior to labeling (iTRAQ® Reagents – 8plex, AB Sciex™, Framingham, MA, USA).

Strong Cation Exchange (SCX) Fractionation of Peptides

Briefly, SCX was carried out using a PolySULFOETHYL™ A Column (PolyLC, Columbia, MD) 5µm particle size of 200mm length × 2.1mm id, 200 Å pore size, on a BioLC HPLC unit (Dionex, Surrey, UK). The chromatogram was monitored through a UV Detector (Dionex/LC Packings, Amsterdam, the Netherlands), at 214 nm.

Mass Spectrometry (MS)

Mass spectrometry was performed using a Q-Star XL Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer, ESI-qQ-TOF-MS/MS (Applied Biosystems; MDS-Sciex) coupled to an online capillary liquid chromatography system (U3000, Thermo, Hemel Hempstead, UK). Peptides were separated on a PepMap C-18 RP capillary column (LC Packings).

16

MS Data Analysis

Peak list conversion was performed using the mascot.dll embedded script (V1.6) coupled with Analyst QS 1.1.1 (ABSCIEX, Framingham, MA, USA). Protein identification and relative quantification was carried out in Phenyx v2.6 (GeneBio S.A., Geneva, Switzerland), using a human protein database. False discovery rate (FDR) was calculated using a decoy database automatically created by reversing the sequences from the target database[70] and was found to be 1%. Figures show exemplar data and samples for iTRAQ were pooled and thus do not include standard errors. For Figure 7, multiple samples from 2D-gels run concurrently have been combined to provide a mean and therefore the standard errors were calculated..

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18

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19

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21

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Legends:

Figure 1: Exemplar images showing the changes in the PD fluid proteome over 4 years on dialysis in a patient with stable membrane function using 2D PAGE

10 PDE samples spanning days 9 to 1410 of peritoneal dialysis of approximately 2 ml volume were precipitated with 3.5 volume of acetone/10% TCA and the pellet was re-solubilised in buffer. A volume equivalent to 60µg of protein was subjected to isoelectric focusing on a pH 4-7 IPG strip (Bio-Rad) using passive rehydration with proteins further separated on a Bio-Rad Dodeca gel system and fractionated overnight. Each gel was recovered and silver stained using the Blum method. Gels were scanned on a Genetools densitometer (transmission), entered into PDQuest software and spot patterns compared. Exemplar images from GFS patient G01-029 (stable membrane function).

Figure 2: Fibrinogen γ-chain (a) and Heparan sulphate proteoglycan (b) are elevated in PD fluid with time on PD in a patient with stable membrane function (exemplar images).

Exemplar images from patient G05-068 (stable membrane function). 10 PD fluid samples were available, ranging from day 30 through to day 3013 on PD that were analysed for spot changes as in figure 1. Protein spots 6001 (a) and 6003 (b) were identified as changing with time on PD by PDQUEST software based on spot volume density. These were excised from the gel and subjected to MALDI-TOF Mass spectrometry. The proteins were identified as fibrinogen γ-chain (a) and heparan sulphate proteoglycan (HSPG) (b). Graphs show a single spot volume density at all 10 timepoints from 1 x 10 gel run. Fibrinogen γ chain gel images depict changes occurring beween 2 ½ & 5 years where as HSPG shows images up to 3 years.

Figure 3: Collagen a(1)I (a) and γ-actin (b) are elevated in a patient with EPS 5 years before EPS diagnosis (exemplar images)

Exemplar images from GFS patients G05-072 (confirmed EPS) and G05-068 (stable membrane

26

function). 5 PD fluid samples were available from patient G05-072, spanning days 1043 to 2868 of

PD. These were matched to 5 samples from patient G05-068, based on time from start of PD and

comparative 2D SDS-PAGE proteomics undertaken as in figure 1. Two spots as identified with a black

arrow in both panels were seen to be consistently elevated in all EPS patients at all time points which

was confirmed by volume densitometric analysis. This protein spots were excised from the gel and

subjected to MALDI-TOF mass spectrometry. The proteins were identified as a fragment of collagen

α1(I) (a) and as γ-actin (b). Of note, the spot identified by a white arrow in panel (a) at 55kDa was

only elevated in this single EPS patient. Graphs show a single spot volume density at all 5 time points

from 1 x 10 gel run with the first 5 bars being the identified spot in the EPS sample and the second 5

bars the stable patient plotted in chronological area.

Figure 4: Complement factors I and B increase in EPS 5 years before diagnosis (exemplar images).

Exemplar images from GFS patients G05-072 (confirmed EPS) and G05-068 (stable membrane

function). 5 PD fluid samples were available from patient G05-072, spanning days 1043 to 2868 of

PD. These were matched to 5 samples from patient G05-068 based on time from start of PD and

comparative 2D SDS-PAGE proteomics performed as figure 1. Two spots that were elevated by

volume densitometry in EPS in this and 3 out of 4 other patient comparison runs were identified as

complement factor I (black arrow) and complement factor B (white arrow).

Figure 5: iTRAQ identifies 4 proteins with altered levels in an EPS patient compared to a matched control.

 Four PD samples from patient G05-072 (confirmed EPS) were matched with 4 PD samples from

patient G05-068 (stable membrane function), by time to finishing dialysis. Each sample was

precipitated in 3 volumes of acetone/10% TCA, re-dissolved & then buffer exchanged into

triethylammonium bicarbonate (TEAB).   50 µg of protein was denatured in tris-(2-

27

carboxyethyl)phosphine and reduced in methylmethane-thiosulfonate prior to trypsin digestion. Each

sample was then labelled with a different isobariq tag using the 8 label iTRAQ system. All samples

were mixed, fractionated by RP-HPLC and proteins identified by MODI-TOF MS. For each of 72

proteins detected, the ratio of each protein was calculated based on the abundance of each isobariq

tag within that protein sample. Using this approach 4 proteins showed consistent trends.

Orosomucoid-1 and α2-HS-glycoprotein chain B were both elevated in the EPS patient some 12

months before diagnosis, while Apolipoprotein A-IV was always low in EPS with a dramatic late drop in

serotransferrin. Data shown represents fold change in the confirmed EPS patient compared to the

patient with stable membrane function and is from a single experimental run.

Figure 6: ProteoMiner allows detection of lower abundance proteins in PD fluid

A PD fluid volume equivalent to 15mg of protein was reduced to 500µl volume using a 3kDa molecular

weight cut off concentrator. 10mg of protein was then subjected to the low abundance protein

enrichment using the Bio-Rad ProteoMiner™ system using 20µl of beads. 100 µg of eluted protein

and that from the original PD fluid samples was precipitated using 3 volume acetone/10% TCA and

comparative analysis performed using 2D SDS PAGE proteomic analysis as in figure 1. The total

number of resolved protein spots was calculated using PDQUEST.

Figure 7: In protein mined PD fluid Intelectin-1 (a) and dermatopontin (b) are elevated in EPS.

From a Greek cohort of PD fluid samples (University of Patras), fluid was taken from 3 patients with

suspected EPS (2 of them confirmed post-portem), 3 patients who had remained on PD for more than

5 years but had no signs of EPS (late controls) and 3 patients with samples taken within the first

month of starting PD (early controls). A volume equivalent to 15mg of protein was concentrated and

subjected to ProteoMiner™ enrichment as in figure 6. Samples were then subjected to 2D SDS-

PAGE analysis as in figure 1, with spots identified as different between the EPS (column 1), late

controls (column 2) and early controls (column 3) identified using PDQUEST. Spot 7203 (a) was

identified as being elevated in all 3 patients with EPS, which was confirmed by subsequent volume densitometry measurement of the spot in all 9 samples. Spot 1001 (b) was identified as being elevated in all 3 patients with EPS and one of the late controls, which was confirmed by subsequent volume densitometry measurement of the spot in all 9 samples. These spots were subsequently excised from the gel and identified by MALDI-TOF as intelectin-1 (a) and Dermatopontin (b). Spot volume densitometry from all 3 patients in each group was averaged with data presented as mean±SEM in the 2 bar graphs. Significance was determined by 1 way ANOVA, * $p < 0.05$, ** $p < 0.01$ compared to early PD control group.

Figure 8: Analysis of pooled samples at four matched time-points using iTRAQ after treatment with Proteominer.

Patient samples were pooled into 4 groups based on time (>24 mths, 12-24 mths, 6-12 mths , <6 mths) to developing EPS or stopping PD. Each pool consisted of equal amounts of protein from each sample available. Post pooling they were processed using ProteoMiner™, iTRAQ labelled, fractionated by RP HPLC and then subjected to tandem MS.

Figure 9: Hierarchical Cluster analysis (a) and heatmap (b) of proteins detected from proteome-mined pooled EPS PD effluent iTRAQ analysis.

The relative contribution of each pooled cohort to the total level of each protein identified was made based on the abundance of each isobariq tag. The proteins detected in each cohort were then used to position each as a hierarchical cluster. E=EPS, N=Normal (stable PD), E1/N1 = >24 mths, E2/N2 = 12-24, E3/N3 = 6-12 mths and E4/N4= <6Mths to EPS or stopping PD **(a)**. The variation in each protein detected in each cohort was then plotted as a heat map (b). Arrows indicate Intellectin-1 and Gelsolin. Data is from a single run.

Figure 10: Relative changes in proteins showing progressive variation with time in pooled

cohorts of EPS patients subjected to protein mining and ITRAQ analysis.

Relative levels of intelectin-1, Gelsolin, Apolipoprotein A-II and Heamaglobin beta subunit were determined by calculating the ratio to the >24-month cohort level in the patients not developing EPS. Data represents ratio to >24month stable patient from a single run of pooled samples.

Table 1: Proteins detectable by iTRAQ in PD fluid

Total proteins identified in PD fluid samples by iTRAQ. Identification is based on at least 3 different identifiable sequences within a protein.

Table 2: Top potential hits for EPS bio markers from 2D PAGE & iTRAQ proteomics

From all 2D SDS-PAGE proteomics using mined and un-mined samples plus iTRAQ studies, proteins that were significantly altered in at least 75% of patients with EPS and less than 10% of non-EPS patients were short-listed as those most likely to have value as prognostic or diagnostic markers of EPS. For 2D gels data shows number of patients showing the change out of total evaluated.

Table 3: Patient Demographics for (a) the GFS and (b) the University of Patras cohorts.

Figures are given as mean (SD), median (range) and percentages as appropriate. For iTRAQ analysis of protein mined samples (**a**), GFS effluent samples were pooled into 4 groups based on time to developing EPS or stopping PD: Group 1; < 6 months prior to EPS or stopping dialysis, Group 2; 6-12 months prior to stopping dialysis, Group 3; 12 to 24 months prior to stopping dialysis and Group 4; greater than 24 months before cessation of dialysis (see also figure 8). Each group consisted of 5 to 7 patient samples.

Comorbidity was documented using the validated Stoke Comorbidity Index, which both categorizes patients into low-risk (score 0), intermediate-risk (score 1–2), and high-risk (score >2) groups and enables analysis by individual comorbid conditions within the index[39].

For the ProteoMiner™ experiments, a single peritoneal fluid sample was obtained from each of nine

30

selected patients from a cohort of Greek PD patients (**b**).

Patients and Methods

Patient Cohorts, Matching and Sample Collection

Patients were drawn from two cohorts, the Global Fluid Study (GFS), an international, multi-centre, prospective, observational cohort study seeking to relate peritoneal membrane function to local and systemic biomarkers as predictors of predefined clinical endpoints (e.g. patient survival, membrane injury)[66], and a smaller single-centre cohort of Greek patients. GFS recruited incident (within first 90 days of PD) and prevalent patients from 10 participating centres from the UK, Korea and Canada between June 2002 and December 2008. Dialysate sampling was from a 4-hour peritoneal equilibration test (PET) using a 2.27% glucose bag, with simultaneous clinical data collected and stored in a purpose built Peritoneal Dialysis Access database (PDDB). The study was conducted adhering to the tenets of the Declaration of Helsinki and informed consent was obtained from all patients. Ethical approval was obtained from the Multi-Centre Research Ethics Committee for Wales covering the UK, whilst local country ethics were obtained for other contributing countries.

For this analysis, EPS patients were identified according to ISPD guidelines[1] in 5 UK centres. For 2D gel proteomics, Global patients were matched primarily on the duration of PD and centre, which in some comparisons required 2 control patients. Closer matching was not possible due to the limited number of patients having prolonged exposure to PD. For ITRAQ proteomics, Global patients were matched based on time to EPS. For pooled studies, 10 EPS patients and 18 with stable membrane function were divided into 4 time segmentsof <6 months, 6 to 12 months, 12 to 24 months and >24 months from EPS or stopping PD (supplemental table 1). Each pool consisted of between 5 and 7 samples which together made up a minimum amount of 18.8mg of starting protein. This protein pool was made up using equivalent protein amounts from all patients contributing to the pool. The

demographic and clinical characteristics of these patients and their controls, including the number of samples analysed and their temporal matching is summarized in Table 3a.

To optimise ProteoMiner™ for use with PDE, experiments requiring larger dialysate volumes, samples from a smaller PD effluent repository based at the University Hospital of Patras, Greece were used. This repository was created in 2009 with the aim to collect samples of peritoneal tissue and PDE, to study potential correlations between effluent markers of inflammation and fibrosis and biopsy findings at catheter placement and removal. The project, was approved by the Ethics Committee of the University Hospital of Patras and informed consent was obtained from incident (scheduled for peritoneal catheter placement) and prevalent patients receiving PD treatment in the same centre. 100ml PDE samples from overnight (8-hour) dwells (Physioneal® 40 Glucose 1.36% w/v, Baxter Healthcare Ltd.) were collected at recruitment (within the first month from PD start for incident patients) and at 6-month intervals thereafter. Samples were also obtained at PD stop, due to kidney transplant or transfer to hemodialysis. At each sampling visit, clinical and demographic data were collected and stored in a database. For the present analysis a single sample from each of nine selected patients was analysed. Selected patients included three incident patients, three prevalent patients with >5 years on PD and stable membrane function and three prevalent patients with suspected EPS based on clinical presentation. In two of the latter, EPS diagnosis was confirmed at autopsy. The third patient was lost to follow up after having been transferred to hemodialysis at another institution. The demographic and clinical characteristics of these patients are summarized in Table 3b.

Sample preparation

All samples were stored in -80° C and thawed for aliquoting and just prior to use in experiments. Total protein was precipitated using 3.5 volumes of acetone

chilled at -20°C and 0.5 volumes of 100%TCA for every volume of sample. Subsequently, samples were centrifuged in a Sigma 3-18 centrifuge (Sigma Laborzentrifugen GmbH, Germany) at 4248 RCF for 40 minutes, at a temperature of 10°C. The protein pellet was re-solubilised in 500µl of buffer containing 8M urea, 2M thiourea, 4% CHAPS, 0.2% (v/v) ampholytes (pH 4−7, 100 mM DTT, 40 mM Tris−base, and 0.0002% bromophenol blue in 18.2MOhm water. To facilitate re-solubilisation, samples were vortexed for 30 seconds and sonicated in a cold water bath for 15 minutes. Following re-solubilisation, protein concentration was determined using the Thermo Scientific Pierce 660nm Protein Assay (Thermo Fisher Scientific Rockford, IL., U.S.A), according to manufacturer's instructions. A volume corresponding to 60µg of protein was increased to a final volume of 350µl by adding solubilisation buffer. This volume was loaded on a pH 4-7 immobilised pH gradient (IPG) strip (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.), covered with 3ml of mineral oil and left overnight to allow complete absorption on the strip.

Isoelectric Focusing

Loaded IPG strips were subjected to isoelectric focusing (IEF) using a PROTEAN IEF system (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.). Default temperature was set to 20°C. A four-step programme was used as follows: Step 1: 250V over 4.5 hours. Step 2: linear increase up to 10,000V over 2.5 hours. Step 3: instant increase up to 10,000V until 50,000 V*h were completed. Step 4: instant decrease down to 500V until 70,000 V*h were completed.

SDS-PAGE

Following isoelectric focusing, IPG strips were bathed in 2% DTT followed by 2.5% iodoacetamide in equilibration buffer for 15 minutes. Equilibration buffer contained 36% (w/v) urea, 20% glycerol, 2% SDS and 0.005M Tris-HCl (pH 8.8). Proteins were subsequently fractionated on a 20 x 23cm 10% polyacrylamide gel

containing 0.1% SDS, 2.5% Rhinohide™ (Invitrogen™), 0.05% APS, 0.025% TEMED

and 0.375M Tris-HCl. After casting the gels in the casting chamber, 1ml of 50:50

water saturated butan-2-ol was added on top of each gel to remove air bubbles and

create a flat surface where the IPG could be applied. 1% overlay agarose (with

Bromophenol Blue to observe dye front) secured the IPG. Gels were allowed to set

for 2 hours, then washed 3 times with ultra-pure water and finally washed with

running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and stored at 4°C

until use. For SDS-PAGE a PROTEAN Plus Dodeca Cell system was used (Bio-Rad

Laboratories Ltd., Hercules, CA, U.S.A.). Voltage was set to 80V for a minimum of 4

hours and then turned down to 40V until electrophoresis was complete.

Gel fixing and silver staining

After electrophoresis, each gel bathed in fixing solution containing 40%

methanol and 10% acetic acid in deionized water for 2 hours. Subsequently gels

were washed with 30% ethanol in water, 3 times for 20 minutes each. For silver

staining, an optimised protocol based on the method described by Blum et al. was

used[67]   First gels bathed in 0.035% sodium thiosulfate for 90 seconds and then

were washed with deionized water 3 times, 20 seconds each. Subsequently, 0.2%

silver nitrate, 0.02% formaldehyde solution was added for 20 minutes and then gels

were washed again 3 times with deionized water, for 20 seconds each. Silver

solution was followed by the developer solution containing 3% sodium carbonate,

0.003% sodium thiosulfate pentahydrate and 0.05% formaldehyde. The developer

solution was decanted after 5 minutes, provided that visualization of protein spots

was clear. Two 30 second washes with water followed before the stop solution

containing 0.5% glycine was added for 10 minutes. Finally, the stop solution was

decanted and gels were washed with water twice, for 30 minutes each time.

Gel Imaging

Gels were scanned on a Genetools densitometer (transmission).   PD Quest v8.0 (Bio-Rad Laboratories Ltd., Hercules, CA, U.S.A.) was used for analysis of the gel images.

Proteome mining

This was undertaken using the Proteominer™ small capacity kit (Biorad, Laboratories Ltd., Hercules, CA, U.S.A.).   A volume of sample corresponding to 15mg of protein was centrifuged using a 3kDa cut-off filter until reduced to 500µl. Subsequently, volumes corresponding to 10mg of protein were mined as per manufacturer's instructions using a ratio of 20µl of beads to 10 mg of protein. Elution with 8M urea/2% CHAPS rehydrated with 5% acetic acid, was followed by a second elution using 4% boiling SDS, 25mM DTT to increase recovery[68]   Finally, protein concentration was determined prior to isoelectric focusing or iTRAQ.

iTRAQ

iTRAQ provides a method for simultaneous protein identification and relative quantification in multiplex format. It is a widely used quantitative proteomic strategy in clinical proteomic research, including for biomarker discovery. During iTRAQ analysis, peptides from each sample (post proteolytic digestion preparation step) are labeled with one of the set of reagents, then combined. The iTRAQ reagents are isobaric and thus a labeled peptide set (for a specific amino acid sequence) will have the same mass in all samples at this stage. Peptide identity and the samples in which it is found is determined by tandem mass spectrometry, which selects a specific set of peptides for fragmentation to yield sequence ions and ions for relative quantification. This data provides information on the relative abundance of a particular peptide across all samples within the experiment. Quantification data is based on unique low mass 'reporter ion' (to each iTRAQ label). Reporter ion intensity information is used to calculate fold change relative to a designated control.

We used an in house statistical analysis pipeline, as detailed in Pham et al. 2010[69] to i) integrate all the peptide measurement data to the protein level and ii) to determine proteins altered in level between samples (p value <0.05). Sample labeling with iTRAQ reagents was performed as described elsewhere[70] and as recommended by the manufacturer. One hundred micrograms of protein from each sample (or pool) was reduced, alkylated then digested with trypsin, prior to labeling (iTRAQ® Reagents – 8plex, AB Sciex™, Framingham, MA, USA).

Strong Cation Exchange (SCX) Fractionation of Peptides

The fractionation was performed as described elsewhere[41]. Briefly, SCX was carried out using a PolySULFOETHYL™ A Column (PolyLC, Columbia, MD) 5 μm particle size of 200 mm length × 2.1 mm id, 200 Å pore size, on a BioLC HPLC unit (Dionex, Surrey, UK). The 60-min gradient was generated between Buffer A (10 mM KH_2PO_4 and 25% acetonitrile, pH 3.0), and Buffer B (10 mM KH_2PO_4, 25% acetonitrile and 500 mM KCl, pH 3.0), and consisted of 100% A for 5 min, 5–30% B for 40 min, 30–100% B for 5 min, 100% B for 5 min and finally 100% A for 5 min. The chromatogram was monitored through a UV Detector (Dionex/LC Packings, Amsterdam, the Netherlands), at 214 nm. Individual SCX fractions were pooled for subsequent nano-LC-MS/MS analysis. Pooled fractions were dried in a vacuum concentrator (Eppendorf, Hamburg, Germany), and stored at −20°C prior to analysis by mass spectrometry.

Mass Spectrometry (MS)

Each peptide fraction was re-dissolved in 20 µl of 0.1% formic acid and 3% acetonitrile and then 6 µl of sample was injected into the nano-LC-ESI-MS/MS system for analysis. Mass spectrometry was performed using a Q-Star XL Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer, ESI-qQ-TOF-MS/MS (Applied Biosystems; MDS-Sciex) coupled to an online capillary liquid chromatography system (U3000, Thermo, Hemel Hempstead, UK). Peptides were separated on a PepMap C-18 RP capillary column (LC Packings), with a constant flow rate of 0.3 µl/min. The LC gradient started with 3% Buffer B (0.1% formic acid in 97% acetonitrile) and 97% Buffer A (0.1% formic acid in 3% acetonitrile) for 3 min, followed by 3–30% Buffer B for 90 min, then 90% Buffer B for 7 min, and finally 3% Buffer B for 8 min. The mass spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 400–1,600 m/z. Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to 3 sec. The two most abundantly charged peptides above a threshold of five counts were selected for MS/MS, and dynamically excluded for 60 sec with a ±50 mmu mass tolerance.

MS Data Analysis

Peak list conversion was performed using the mascot.dll embedded script (V1.6) coupled with Analyst QS 1.1.1 (ABSCIEX, Framingham, MA, USA) with MS/MS group summations and the iTRAQ region deisotoping removed. Protein identification and relative quantification was carried out in Phenyx v2.6 (GeneBio S.A., Geneva, Switzerland), using a human protein database. General search parameters allowed for MS and MS/MS tolerance up to 0.1 Da and one missed cleavage. Fixed protein modifications included iTRAQ lysine and iTRAQ N-terminus (+304 Da) and methyl-thiol of cysteines (+46 Da), variable modifications were oxidation of methionine (+16 Da). The acceptance parameters for peptide

identification were peptide length ≥6, z-score ≥5.0 and p-value ≤1.0 e-4. False

discovery rate (FDR) was calculated using a decoy database automatically created

by reversing the sequences from the target database[71] and was found to be 1%.

Bias normalization for whole proteome analyses were also performed by correcting

the bias median ratio of each comparison toward unity. Figures show exemplar data

and samples for iTRAQ were pooled and thus do not include standard errors. For

Figure 7, multiple samples from 2D-gels have been combined to provide a mean and

therefore the standard error was calculated.

Supplemental Table 1: Patient sample matching for 2D gel comparisons

Patient Code EPS vs Stable PD	Days from starting PD				
	Point 1	Point 2	Point 3	Point 4	Point 5
G05-072 vs. G05-068	1043 vs 1089	1448 vs 1490	2429 vs 2430	2538 vs 2564	2868 vs 2013
G05-134 vs G01-029	9 vs 9	181 vs 203	376 vs 203	933 vs 870	-
G03-016 vs G03-022	772 vs 828	870 vs 869	1141 vs 1136	1297 vs 1213	-
G01 001 vs G01 030	1204 vs 1142	1567 vs 1497	2177 vs 2241	2350 vs 2439	-
G01-001 vs G01-15	774 vs 785	960 vs 953	1142 vs 1165	1346 vs 1347	-
G05-018 vs G03-006	34 vs 90	258 vs 294	435 vs 447	664 vs 665	-
G05-72 vs G0157	2009 vs 2170	2429 vs 2382	2638 vs 2501	2868 vs 2737	-

EPS and stable PD patient samples were matched at up to 5 time points from the date of starting PD for 2D Gel proteomic analysis. Each comparison shows the day that the sample was collected from the EPS patient versus the day from the stable PD patient

Supplemental table 2: Breakdown of EPS and control sample pooling for iTRAQ analysis on protein mined PDE

Patient Code	Condition	<6 months	6-12 Mths	12-24 Mths	>24 Months	Number of Time points
G05-072	EPS	Y	Y	Y	Y	4
G05-134	EPS	Y		Y		2
G03-016	EPS	Y		Y	Y	3
G05-092	EPS	Y				1
G05-010	EPS	Y	Y			2
G01-008	EPS		Y	Y	Y	3
G05-029	EPS		Y			1
G05-018	EPS		Y	Y		2
G01-001	EPS				Y	1
G02-052	EPS				Y	1
EPS pooled @ each time point		**5**	**5**	**5**	**5**	
G05-042	Stable PD	Y				1
G05-002	Stable PD	Y				1
G03-006	Stable PD	Y				1
G05-093	Stable PD	Y	Y			2
G03-021	Stable PD	Y		Y		2
G05-050	Stable PD	Y				1
G05-090	Stable PD		Y			1
G05-123	Stable PD		Y			1
G01-011	Stable PD		Y	Y	Y	3
G05-077	Stable PD		Y			1
G05-119	Stable PD			Y		1
G05-026	Stable PD			Y		1
G03-018	Stable PD			Y	Y	2
G05-085	Stable PD			Y		1
G05-041	Stable PD				Y	1
G01-031	Stable PD				Y	1
G05-069	Stable PD				Y	1
G01-069	Stable PD				Y	1
G02-066	Stable PD				Y	1
Stable pooled @ each time point		**6**	**5**	**6**	**7**	

EPS samples were pooled by time to developing EPS or stopping PD with stable function into cohorts of <6 months, 6-12 months, 12-24 months and .24 months. Each pool contained samples that together gave a minimum of 18.8 mg of protein. All patients (5 to 7) in a pool contributed an equal amount of protein. Some patients contributed to all pools, and some just 1. Y denotes inclusion in the pool. GXX denotes the centre and the following number the patient at that centre.

Figure 1: Exemplar images showing the changes in the PD fluid proteome over 4 years on dialysis in a patient with stable membrane function using 2D PAGE

10 PDE samples spanning days 9 to 1410 of peritoneal dialysis of approximately 2 ml volume were precipitated with 3.5 volume of acetone/10% TCA and the pellet was re-solubilised in buffer. A volume equivalent to 60μg of protein was subjected to isoelectric focusing on a pH 4-7 IPG strip (Bio-Rad) using passive rehydration with proteins further separated on a Bio-Rad Dodeca gel system and fractionated overnight. Each gel was recovered and silver stained using the Blum method. Gels were scanned on a Genetools densitometer (transmission), entered into PDQuest software and spot patterns compared. Exemplar images from GFS patient G01-029 (stable membrane function).

Figure 1
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Figure 2: Fibrinogen γ-chain (a) and Heparan sulphate proteoglycan (b) are elevated in PD fluid with time on PD in a patient with stable membrane function (exemplar images).

Exemplar images from patient G05-068 (stable membrane function). 10 PD fluid samples were available, ranging from day 30 through to day 3013 on PD that were analysed for spot changes as in figure 1. Protein spots 6001 (a) and 6003 (b) were identified as changing with time on PD by PDQUEST software based on spot volume density. These were excised from the gel and subjected to MALDI-TOF Mass spectrometry. The proteins were identified as fibrinogen γ-chain (a) and heparan sulphate proteoglycan (HSPG) (b). Graphs show a single spot volume density at all 10 timepoints from 1 x 10 gel run. Fibrinogen γ chain gel images depict changes occurring beween 2 ½ & 5 years where as HSPG shows images up to 3 years.
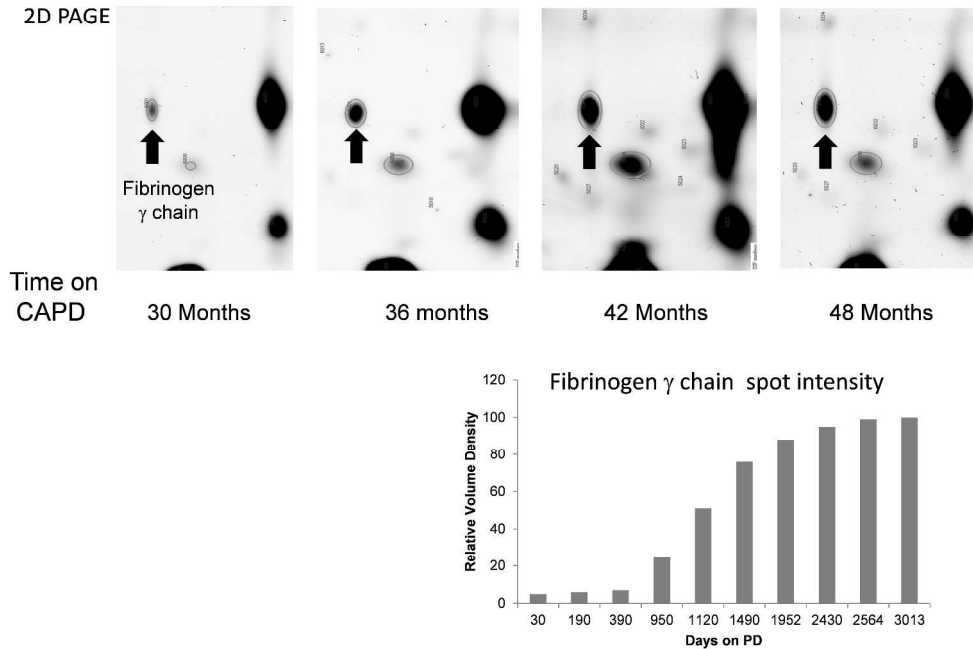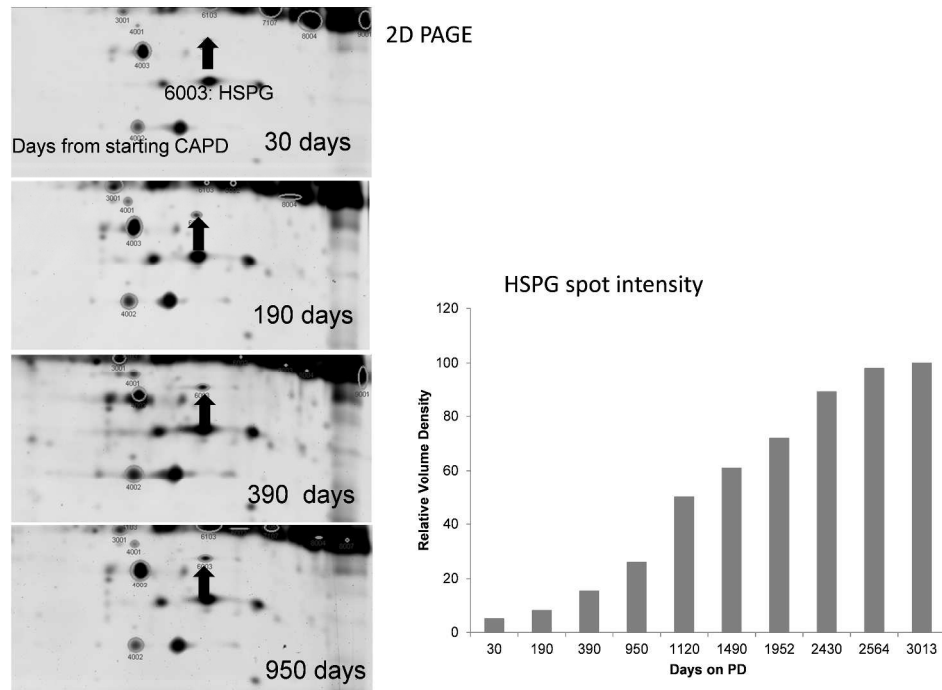
Figure 2a
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Figure 2: Fibrinogen γ-chain (a) and Heparan sulphate proteoglycan (b) are elevated in PD fluid with time on PD in a patient with stable membrane function (exemplar images).
Exemplar images from patient G05-068 (stable membrane function). 10 PD fluid samples were available, ranging from day 30 through to day 3013 on PD that were analysed for spot changes as in figure 1. Protein spots 6001 (a) and 6003 (b) were identified as changing with time on PD by PDQUEST software based on spot volume density. These were excised from the gel and subjected to MALDI-TOF Mass spectrometry. The proteins were identified as fibrinogen γ-chain (a) and heparan sulphate proteoglycan (HSPG) (b). Graphs show a single spot volume density at all 10 timepoints from 1 x 10 gel run. Fibrinogen γ chain gel images depict changes occurring beween 2 ½ & 5 years where as HSPG shows images up to 3 years.
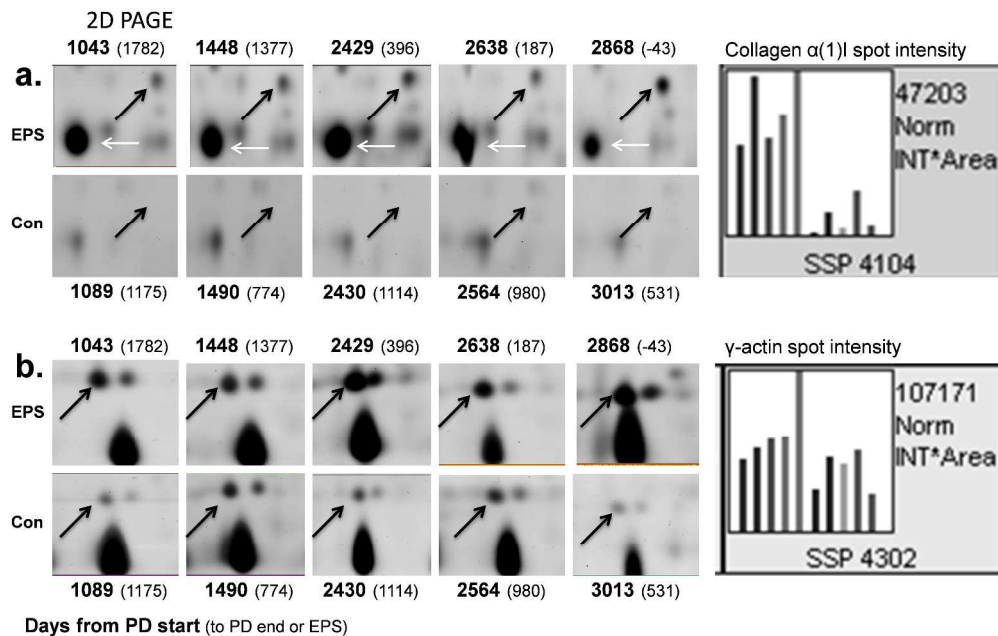
Figure 2b
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Figure 3: Collagen a(1)I (a) and γ-actin (b) are elevated in a patient with EPS 5 years before EPS diagnosis (exemplar images)
Exemplar images from GFS patients G05-072 (confirmed EPS) and G05-068 (stable membrane function). 5 PD fluid samples were available from patient G05-072, spanning days 1043 to 2868 of PD. These were matched to 5 samples from patient G05-068, based on time from start of PD and comparative 2D SDS-PAGE proteomics undertaken as in figure 1. Two spots as identified with a black arrow in both panels were seen to be consistently elevated in all EPS patients at all time points which was confirmed by volume densitometric analysis. This protein spots were excised from the gel and subjected to MALDI-TOF mass spectrometry. The proteins were identified as a fragment of collagen α1(I) (a) and as γ-actin (b). Of note, the spot identified by a white arrow in panel (a) at 55kDa was only elevated in this single EPS patient. Graphs show a single spot volume density at all 5 time points from 1 x 10 gel run with the first 5 bars being the identified spot in the EPS sample and the second 5 bars the stable patient plotted in chronological area.

Figure 3a, Figure 3b
1270x952mm (120 x 120 DPI)

EPS

1043 (1782)   **1448** (1377)   **2429** (396)   **2638** (187)   **2868** (-43)

Control

1089 (1175)   **1490** (774)   **2430** (1114)   **2564** (980)   **3013** (531)

Days from PD start (to PD end or EPS)

Complement Factor I (black arrow)

Complement Factor B (white arrow)

Figure 4: Complement factors I and B increase in a patient with EPS 5 years before diagnosis (exemplar images).‖ ⊦ Exemplar images from GFS patients G05-072 (confirmed EPS) and G05-068 (stable membrane function). 5 PD fluid samples were available from patient G05-072, spanning days 1043 to 2868 of PD. These were matched to 5 samples from patient G05-068 based on time from start of PD and comparative 2D SDS-PAGE proteomics performed as figure 1. Two spots that were elevated by volume densitometry in EPS in this and 3 out of 4 other patient comparison runs were identified as complement factor I (black arrow) and complement factor B (white arrow).‖ ⊦

Figure 4

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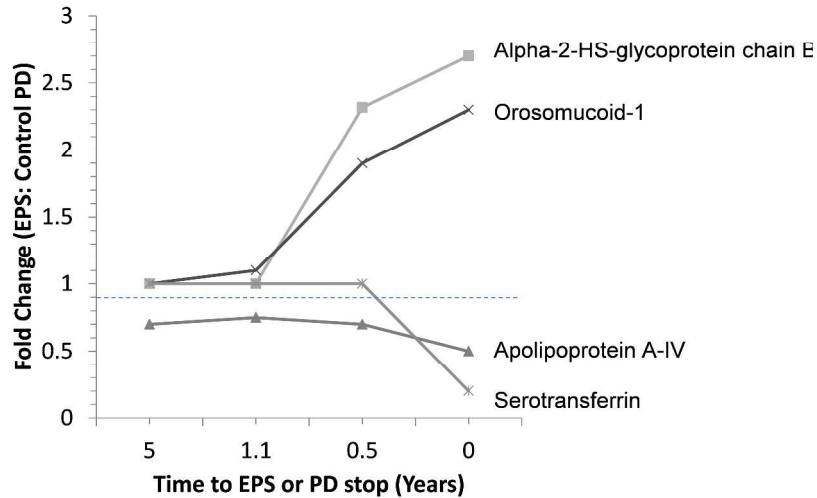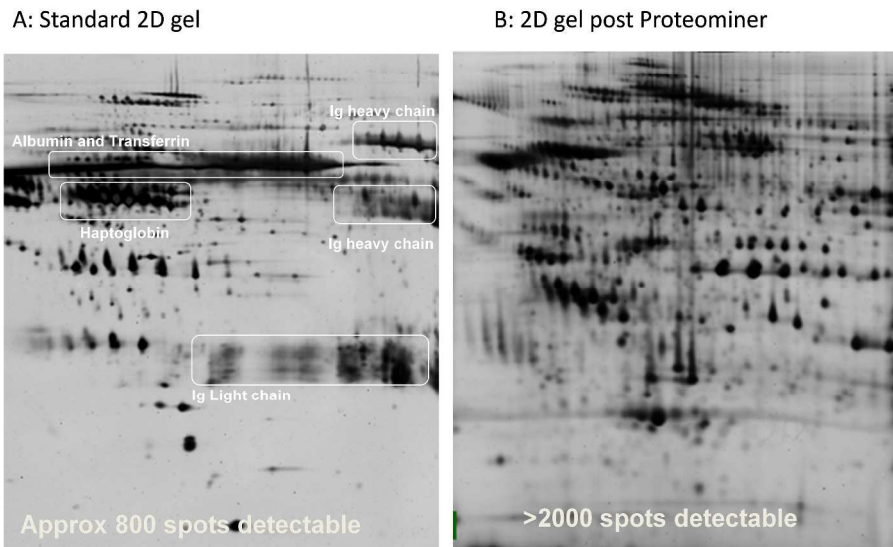21
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Figure 5: iTRAQ identifies 4 proteins with altered levels in an EPS patient compared to a matched control. Four PD samples from patient G05-072 (confirmed EPS) were matched with 4 PD samples from patient G05-068 (stable membrane function), by time to finishing dialysis. Each sample was precipitated in 3 volumes of acetone/10% TCA, re-dissolved & then buffer exchanged into triethylammonium bicarbonate (TEAB). 50 μg of protein was denatured in tris-(2-carboxyethyl)phosphine and reduced in methylmethane-thiosulfonate prior to trypsin digestion. Each sample was then labelled with a different isobariq tag using the 8 label iTRAQ system. All samples were mixed, fractionated by RP-HPLC and proteins identified by MODI-TOF MS. For each of 72 proteins detected, the ratio of each protein was calculated based on the abundance of each isobariq tag within that protein sample. Using this approach 4 proteins showed consistent trends. Orosomucoid-1 and α2-HS-glycoprotein chain B were both elevated in the EPS patient some 12 months before diagnosis, while Apolipoprotein A-IV was always low in EPS with a dramatic late drop in serotransferrin. Data shown represents fold change in the confirmed EPS patient compared to the patient with stable membrane function and is from a single experimental run.

Figure 5
1270x952mm (120 x 120 DPI)

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Figure 6: ProteoMiner™ allows detection of lower abundance proteins in PD fluid
A PD fluid volume equivalent to 15mg of protein was reduced to 500µl volume using a 3kDa molecular weight cut off concentrator. 10mg of protein was then subjected to the low abundance protein enrichment using the Bio-Rad ProteoMiner™ system using 20µl of beads. 100 µg of eluted protein and that from the original PD fluid samples was precipitated using 3 volume acetone/10% TCA and comparative analysis performed using 2D SDS PAGE proteomic analysis as in figure 1. The total number of resolved protein spots was calculated using PDQUEST.

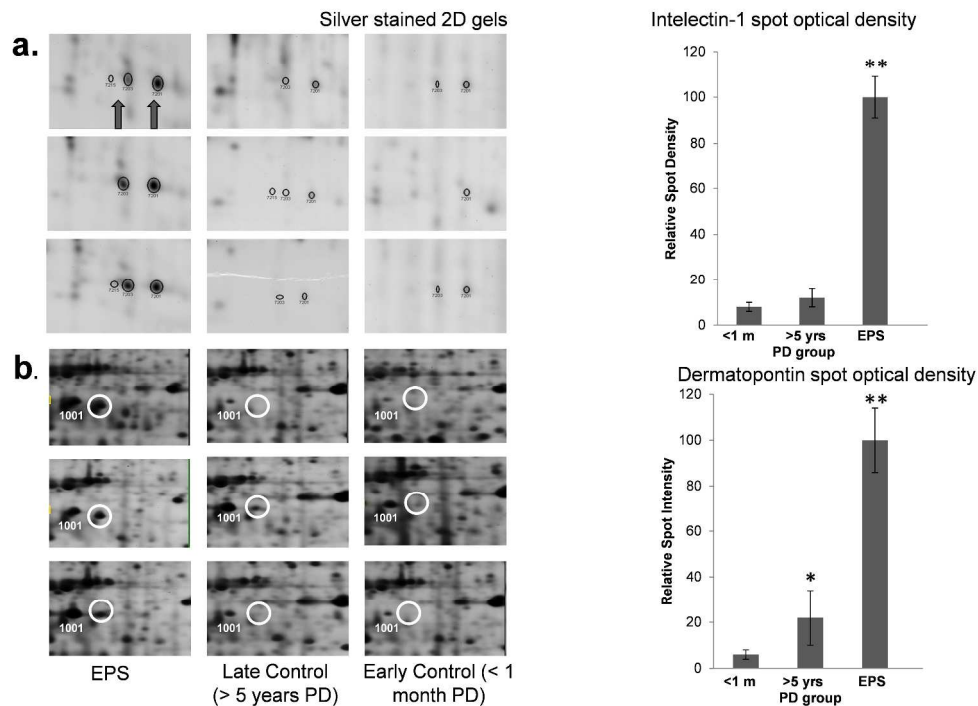Figure 6A, Figure 6B
1270x952mm (120 x 120 DPI)

Figure 7: In protein mined PD fluid Intelectin-1 (a) and dermatopontin (b) are elevated in EPS. From a Greek cohort of PD fluid samples (University of Patras), fluid was taken from 3 patients with suspected EPS (2 of them confirmed post-portem), 3 patients who had remained on PD for more than 5 years but had no signs of EPS (late controls) and 3 patients with samples taken within the first month of starting PD (early controls). A volume equivalent to 15mg of protein was concentrated and subjected to ProteoMiner™ enrichment as in figure 6. Samples were then subjected to 2D SDS-PAGE analysis as in figure 1, with spots identified as different between the EPS (column 1), late controls (column 2) and early controls (column 3) identified using PDQUEST. Spot 7203 (a) was identified as being elevated in all 3 patients with EPS, which was confirmed by subsequent volume densitometry measurement of the spot in all 9 samples. Spot 1001 (b) was identified as being elevated in all 3 patients with EPS and one of the late controls, which was confirmed by subsequent volume densitometry measurement of the spot in all 9 samples. These spots were subsequently excised from the gel and identified by MALDI-TOF as intelectin-1 (a) and Dermatopontin (b). Spot volume densitometry from all 3 patients in each group was averaged with data presented as mean±SEM in the 2 bar graphs. Significance was determined by 1 way ANOVA, * $p<0.05$, ** $p<0.01$ compared to early PD control group.

Figure 7a, Figure 7b
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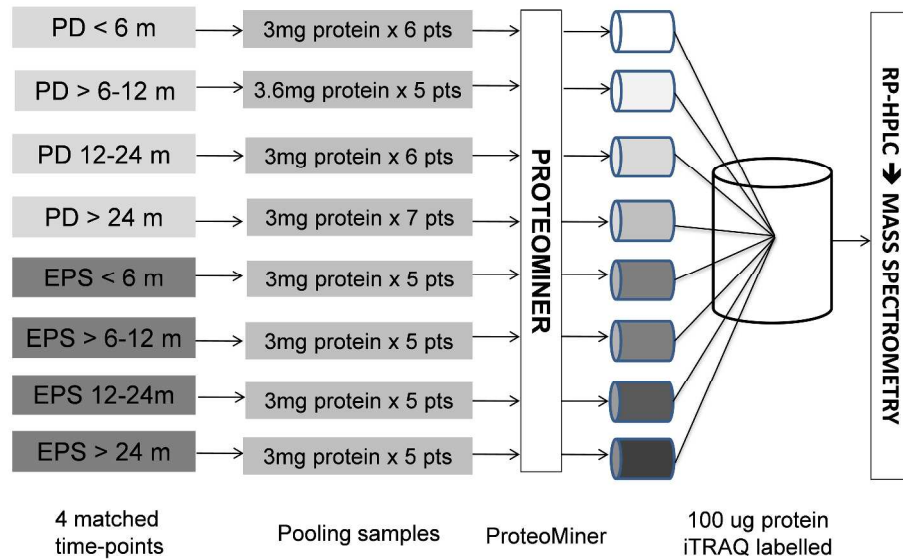
Figure 8: Analysis of pooled samples at four matched time-points using iTRAQ after treatment with Proteominer™.
Patient samples were pooled into 4 groups based on time (>24 mths, 12-24 mths, 6-12 mths , <6 mths) to developing EPS or stopping PD. Each pool consisted of equal amounts of protein from each sample available. Post pooling they were processed using ProteoMiner™, iTRAQ labelled, fractionated by RP HPLC and then subjected to tandem MS.

Figure 8
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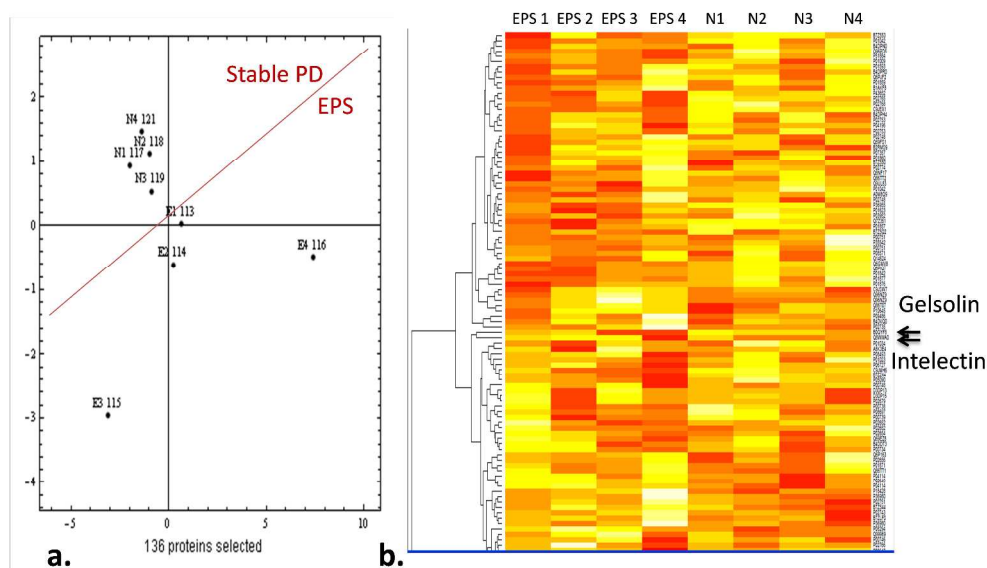
Figure 9: Hierarchical Cluster analysis (a) and heatmap (b) of proteins detected from proteome-mined pooled EPS PD effluent iTRAQ analysis.
The relative contribution of each pooled cohort to the total level of each protein identified was made based on the abundance of each isobariq tag. The proteins detected in each cohort were then used to position each as a hierarchical cluster. E=EPS, N=Normal (stable PD), E1/N1 = >24 mths, E2/N2 = 12-24, E3/N3 = 6-12 mths and E4/N4= <6Mths to EPS or stopping PD (a). The variation in each protein detected in each cohort was then plotted as a heat map (b). Arrows indicate Intellectin-1 and Gelsolin. Data is from a single run.

Figure 9a, Figure 9b
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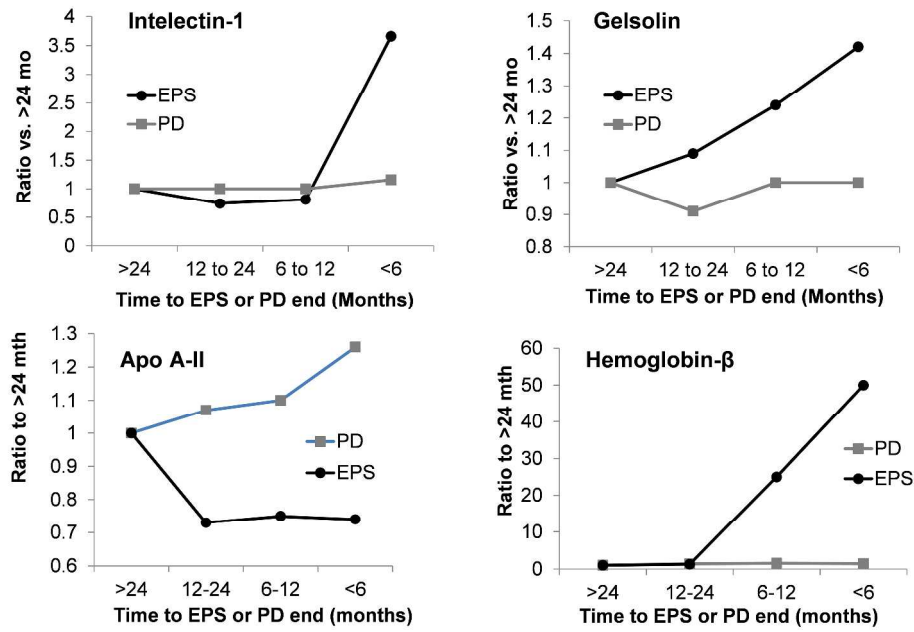
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Figure 10: Relative changes in proteins showing progressive variation with time in pooled cohorts of EPS patients subjected to protein mining and ITRAQ analysis.
Relative levels of intelectin-1, Gelsolin, Apolipoprotein A-II and Heamaglobin beta subunit were determined by calculating the ratio to the >24-month cohort level in the patients not developing EPS. Data represents ratio to >24month stable patient from a single run of pooled samples.

Figure 10
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Table 1: Proteins detectable by iTRAQ in whole proteome P

Serum albumin

Serotransferrin (Transferrin)

cDNA FLJ78387

Short peptide from AAT (SPAAT)

Haptoglobin beta chain

Fibrinogen alpha chain, isoform CRA_c

Apolipoprotein A-I(1-242)

Cyclosporin A transporter 1

Trypstatin

Apolipoprotein A-IV (Apo-AIV) (ApoA-IV)

Alpha-1-acid glycoprotein 1 (AGP 1) (OMD 1)

Complement C3c alpha' chain fragment 2

Ig gamma-3 chain C region

Ig alpha-1 chain C region

Alpha-2-HS-glycoprotein chain B

Hemopexin

Alpha-1-antichymotrypsin His-Pro-less

Fibrinogen gamma chain, isoform CRA_a

Alpha-2-macroglobulin (Alpha-2-M)

Ig kappa chain C region

Transthyretin

HCG1778670

Retinol binding protein 4, plasma, isoform CRA_a

cDNA FLJ53327, highly similar to Gelsolin

Zinc-alpha-2-glycoprotein (Zn-alpha-2-glycoprotein)

Beta-2-microglobulin form pI 5.3

Ig gamma-2 chain C region

Fibrinopeptide A

Alpha-1B-glycoprotein

Alpha-1-acid glycoprotein 2 (AGP 2) (OMD 2)

Complement component 4A (Rodgers blood group)

cDNA FLJ53691, highly similar to Serotransferrin

Fibrinogen beta chain, isoform CRA_e

Low molecular weight growth-promoting factor

Alpha-2-HS-glycoprotein chain B

Complement factor H

LVV-hemorphin-7

cDNA FLJ51265, similar to Beta-2-glycoprotein 1

Putative uncharacterized protein AZGP1

Complement factor B Bb fragment

Ig kappa chain V-III region NG9

Leucine-rich alpha-2-glycoprotein (LRG)

Ig kappa chain V-III region CLL

Histidine-rich glycoprotein, isoform CRA_a

Cystatin-C

Complement component C9b

Myosin-reactive immunoglobulin heavy chain variable region

Complement factor D

Ig kappa chain V-I region EU

ᵖD fluid

Vitamin D-binding protein (DBP) (VDB)

Antithrombin

Cryocrystalglobulin CC1 kappa light chain variable region

Cryocrystalglobulin CC2 lambda light chain variable region

cDNA FLJ37971 fis, clone CTONG2009958, highly similar to CERULOPLASMIN (EC 1.16.3.1)

cDNA FLJ39644 fis, clone SMINT2004037, highly similar to CHLORIDE CHANNEL PROTEIN 7

cDNA FLJ52255, highly similar to Angiotensinogen

Thrombin heavy chain [CHAIN 1]

Apolipoprotein C-III (Apo-CIII) (ApoC-III)

Ugl-Y3

Inter-alpha-trypsin inhibitor heavy chain H1 (Inter-alpha-inhibitor heavy chain 1) (ITI heavy chain H1) (ITI-HC1) (SHAP)

Prostaglandin-H2 D-isomerase (PGD2 synthase) (PGDS2) (PGDS)

IGL@ protein

Nuclear fragile X mental retardation-interacting protein 2 (82-FIP)

Full-length cDNA clone CS0DD006YL02 of Neuroblastoma of Homo sapiens (human)

Zinc finger protein 527

Uncharacterized protein C7orf62

Kelch-like protein 3 [ISOFORM C]

Myosin-reactive immunoglobulin light chain variable region

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Table 2: Top potential hits for EPS bio markers

	Change in EPS PDE			
	2D Gel	2D Gel	ITRAQ	ITRAQ
	FP	MP	FP	MP
γ–Actin	↑			
Collagen(α1) 1	↑			
α1 antitrypsin	↓		↓	
α-2-HS-glycoprotein B			↑	↑
Serotransferrin			↑	
Complement C4B			↑	
Complement B	↑			↑
Complement I	↑			↑
Apolipoprotein A-IV	↓		↓	↓
Orosomucoid-1			↑	↑
Intelectin-1		↑		↑
Dermatopontin		↑		
RBP-4		↑		
Gelsolin				↑
Haemaglobin b				↑
Apolipoprotein A-II				↓

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s from 2D PAGE & iTRAQ proteomics

Frequency of occurrence in 2D gels (times of change)

6/6 patients (early./ elevated >3 yrs before EPS)

6/6 patients (early / > 3 yrs before EPS)

3/6 patients plus iTRAQ (slow reduction with time -v low at EPS)

NA. (mid/ 6 -12 months before EPS)

NA   (early / > 12 months before EPS)

NA   (early / > 12 months before EPS)

 5/6 patients (early/elevated upto 6 years before EPS

5/6 patients (early /elevated up to 6 years before EPS

3/6 plus iTRAQ (late / from 12 months . -80% at EPS)

NA. (Early / > 1 yr before EPS)

3/3 patients & pooled (late/within 6 months of EPS)

3/3 patients (unknown)

3/3 patients   (unknown)

Pooled. (Mid / elevated up to 1 year before EPS)

Pooled (late / elevated up to 12 months before EPS)

Pooled (late/ low for up to 12 mths before EPS

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Table 3a: Global Fluid Study cohort - Demographic Data

| | 2D Gels | | iTRAQ pool | | | |
| | | | Group 1 | | Group 2 | |
	EPS (6 patients, 33 samples)	Control (6 patients, 33 samples)	EPS (5 patients, 5 samples)	Control (6 patients, 6 samples)	EPS (5 patients, 5 samples)	Control (5 patients, 5 samples)
Age (years)	54.8 (15.4)	51.8 (16.5)	45.6 (12.4)	55.0 (14.4)	48.5 (16.3)	54.3 (11.2)
Male Gender	66.70%	66.70%	60%	17%	40%	60%
Comorbidity (Low/Medium/High)	50.0/50.0/0%	50/33.3/16.7%	60.0/40.0/0%	50.0/33.3/16.7%	60.0/40.0/0%	60.0/0/40.0%
Previous peritonitis episodes	0 (0-4)	1 (0-4)	1 (0-6)	2 (0-6)	2 (0-5)	2 (0-3)
Months From PD Start	35.2 (26.0)	33.9 (25.2)	55.1 (24.8)	72.4 (51.2)	73.3 (44.2)	42.6 (15.8)
Months Until PD End	29.5 (21.9)	30.4 (16.7)	1.8 (3.5)	1.8 (0.6)	8.9 (2.2)	8.8 (2.3)
Urine volume (mls)	663 (0-2128)	816 (0-2092)	60 (0-120)	0 (0-940)	170 (0-1322)	120 (0-1508)
Icodextrin Usage	36.40%	26.50%	40%	66.70%	20%	24%
APD usage	21.20%	30.30%	60.00%	80.00%	40%	80%
Dialysate IL-6 (pg/ml)	13.9 (0.1-134.2)	7.0 (0.0-87.8)	75.9 (24.1-134.2)	24.8 (10.1-45.1)	10.7 (2.7-79.8)	12.9 (2.4-52.9)
Plasma IL-6 (pg/ml)	3.8 (0.5-54.7)	1.1 (0.2-4.0)	3.9 (0.7-11.1)	4.1 (0.6-8.8)	3.3 (0.9-9.6)	4.2 (4.1-13.9)
Serum Albumin (g/L)	33.9 (8.2)	36.9 (4.7)	30.0 (10.2)	33.4 (5.5)	35.0 (5.2)	35.8 (3.6)
D/P Cr	0.72 (0.14)	0.69 (0.12)	0.88 (0.08)	0.89 (0.15)	0.92 (0.19)	0.81 (0.14)

ed samples

	Group 3		Group 4	
	EPS (5 patients, 5 samples)	**Control** (6 patients, 6 samples)	**EPS** (5 patients, 6 samples)	**Control** (7 patients, 7 samples)
	48.8 (17.0)	53.1 (18.4)	47.4 (20.8)	60.5 (19.8)
	80%	83.00%	40%	71%
	40.0/60.0/0%	33.3/50.0/16.7%	40.0/60.0/0%	57.1/42.9/0%
	1 (0-4)	2 (0-4)	0.5 (0-2)	1 (0-4)
	38.6 (29.4)	31.5 (17.6)	30.1 (24.6)	32.8 (22.4)
	15.5 (8.9)	15.6 (2.0)	47.9 (11.9)	47.9 (8.6)
	295 (0-1570)	604 (0-1160)	550 (0-2004)	1216 (0-1305)
	60.00%	68.20%	33.30%	79.20%
	40%	66.60%	0%	0.00%
	14.6 (7.7-1902.9)	42.5 (0.7-116.1)	10.2 (4.6-45.1)	4.3 (2.9-13.8)
	4.4 (0.5-31.2)	2.0 (0.6-12.6)	3.4 (0.9-10.1)	1.0 (0.0-5.4)
	29.8 (10.6)	35.7 (4.7)	33.3 (9.2)	38.8 (3.9)
	0.74 (0.12)	0.83 (0.08)	0.70 (0.14)	0.64 (0.09)

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Table 3b: University of Patras cohort - Demographic Data

	EPS (n=3)	Controls (n=3)	New starters (n=3)
Age (years)	59 (30-64)	56 (50-80)	45 (43-60)
Male Gender	67%	33%	33%
Comorbidity (Low/Medium/High)	67/0/33%	67/33/0%	33/67/0%
Previous peritonitis episodes	1 (0-4)	0	0
Months From PD Start	108 (61-178)	85 (68-97)	<1
Urine volume (mls)	0 (0-200)	800 (500-1000)	1000 (600-1700)
Icodextrin Usage	67%	33%	0%
APD usage	33%	33%	33%
Serum Albumin (g/L)	32 (29-33)	34 (32-38)	40 (36-40)
D/P Cr	0.89 (0.74-0.91)	0.62 (0.58-0.76)	0.72 (0.67-0.73)

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