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Proof-of-principle study to detect metabolic changes in peritoneal dialysis effluent in patients who develop encapsulating peritoneal sclerosis

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Abstract

Background. Prolonged peritoneal dialysis (PD) therapy can result in the development of encapsulating peritoneal sclerosis (EPS), characterized by extensive sclerosis of the peritoneum with bowel adhesions often causing obstruction.

Methods. As a proof-of-principle study, holistic profiling of endogenous metabolites has been applied in a prospective collection of PD effluent collected in multiple UK renal centres over 6 years in order to investigate metabolic differences in PD effluent between PD therapy patients who later developed clinically defined EPS ($n = 11$) and controls, who were matched for PD vintage, age and gender ($n = 11$).

Results. 'Fit-for-purpose' analytical methods employing gas chromatography–mass spectrometry (MS), direct injection MS and quality control samples were developed and validated. These methods were applied in a proof-of-principle study to define metabolic differences in PD effluent related to subsequent development of EPS. Changes in amino acids, amines and derivatives, short-chain fatty acids and derivatives and sugars were observed prior to EPS developing, and changes in the metabolomic profiles could be detected.

Conclusion. There is potential for applying metabolic profiles to identify patients at risk of developing EPS although long-term prospective studies with larger patient cohorts are required.

Keywords: EPS; GLOBAL fluid Study; Metabolomics

Introduction

The clinical complications of prolonged peritoneal dialysis (PD) therapy have become more evident in the last decade as increasing numbers of patients remain on PD for >5 years. The most serious complication of PD is the development of encapsulating peritoneal sclerosis (EPS). EPS is characterized by extensive sclerosis of the peritoneum with bowel adhesions often causing obstruction. In the most severe cases, the bowel becomes enveloped in a fibrotic cocoon resulting in severe gastrointestinal (GI) symptoms [1]. EPS is definitively confirmed by surgery though suspicion of EPS is aroused by the presence of gastroenterological symptoms including abdominal pain, vomiting, weight loss, absent bowel sounds and palpable intestinal mass [2]. On presentation, it is common to find a history of chronic GI symptoms resulting in progressive weight loss and declining nutritional status [3]. Often, EPS may be associated with a failing peritoneal membrane which can be defined by the peritoneal equilibrium test (PET) although this is not a definitive test and some patients will develop membrane failure and not progress to EPS. Some recent data have shown that membrane deterioration precedes the development of EPS [4]. The clinical diagnosis of EPS is difficult with the lack of a definitive biochemical test that can be applied to biofluids which will confirm the onset and progression of EPS. Recently, a retrospective study highlighted that higher interleukin (IL)-6 levels and lower CA125 levels (a putative indicator of mesothelial mass/viability, usually associated with reduced membrane function) were found in the PD effluent of patients who subsequently developed EPS [5]. The study of changes in the

metabolomes of biofluids or tissues has not been previously reported in the context of PD and the application of metabolomics may have significant advantages. Early detection of EPS would be extremely helpful in the clinical management and indeed in the development of preventative strategies given that the majority of cases culminate in bowel obstruction and require surgical attention [6]. A better understanding of the molecular pathophysiological mechanisms related to predisposition to and progression of EPS are required to identify potential targets for therapeutic interventions.

The metabolome is defined as the quantitative collection of metabolites in a biological system. The quantitative collection and interaction of biochemicals (genes, transcripts, proteins and metabolites) and the environment in biological systems define the phenotype of that system and the metabolome is biochemically closest to the phenotype. In biological research, it is becoming clear that the study of individual components of the system is not providing robust mechanisms related to the operation of these systems. Instead, a holistic approach to study a large number of components and, more importantly, the interaction of these components is required to define the biological operation of the system. The study of system-wide properties is defined as systems biology [7, 8]. The holistic study of the metabolome (defined as metabolomics) offers a number of advantages including low-cost and high-throughput experiments [9–12]. The interaction of metabolites with other metabolites (metabolism), proteins (allosteric effects and post-translation modifications) and transcripts (riboswitches) all provide regulation of and signalling in biological systems. Phenotypic differences related to age, lifestyle, environmental factors and disease have all been observed with high sensitivity in the biofluid and tissue metabolomes of humans [9, 13, 14]. Metabolomics is typically applied in a holistic manner for knowledge discovery and inductive reasoning [15], followed by further targeted and validation studies, focussed on a small number of metabolites [9].

To date, no studies have looked at the metabolic profiles of PD effluent. In effect, the effluent can be described as a metabolic footprint of the patients cellular metabolism and the impact of the process of dialysis [10]. Metabolic footprinting has been applied to the study of other diseases including pre-eclampsia [16], the effluent defines the metabolites consumed from and secreted into the effluent from the tissue or cell in close proximity.

In this investigation, we aimed, in a proof-of-principle study, to define (i) whether current metabolomic strategies are appropriate for the study of PD effluent and (ii) whether metabolic differences in PD effluent relating to the risk of progression to EPS can be detected. Over the last 6 years, the UK limb of the GLOBAL Fluid Study has prospectively collected and stored PD effluent (and plasma) on up to 600 PD patients. Within this prospective cohort, 11 cases (prevalence of 1.8%) of clinically defined EPS have subsequently occurred. The prospective nature of the collection has allowed the investigation of potential metabolomic biomarkers associated with EPS to be investigated in samples of effluent obtained prior to EPS symptoms being observed. Our hypothesis was that metabolic changes would be detectable prior to diagnosis of EPS. If confirmed, metabolic patterns might aid the understanding of the pathophysiology of EPS and provide a metabolic profile that identifies patients at risk. If this proof-of-principle study was successful, then further large-scale validation studies would be justified.

Materials and methods

Patients and sample collection

All samples were collected after acquisition of ethical approval. Following informed consent into the GLOBAL Fluid Study, peritoneal effluent samples were collected from a cross-sectional cohort of PD patients in a prospective study between 2002 and 2008. The Global PD samples were taken at the time of the PET, after a 4-h dwell time, and were frozen at a temperature of −80°C, within 24 h. Over a 6-year follow-up period, 11 patients were identified as having subsequently developed EPS following recruitment into the study.

Diagnosis was centred around functional (symptoms) and structural (scans) criteria according to International Society of Peritoneal Dialysis (IPSD) guidelines. The diagnosis of EPS in all cases was based on clinical suspicion confirmed with, primarily, radiologic findings (computerized tomography scans). Pathologic confirmation was obtained at the time of laporotomy for management or for catheter removal.

A set of control subjects were retrospectively selected from the GLOBAL database (PDDB), matched for PD vintage, age, gender and body mass index and who had not been diagnosed with EPS. Only the first sample collected (usually at the start of PD for incident patients or at the time of entry into GLOBAL for prevalent patients) was used for this analysis. Samples of PD fluid from unused dialysate fluids [Dianeal (1.36, 2.27 and 3.86), Physioneal and Extraneal] were analysed to provide baseline metabolic profiles.

Chemicals

All chemicals and solvents applied were of high-performance liquid chromatography (HPLC) grade or higher and were supplied by Sigma–Aldrich (Gillingham, UK) or Acros Chemicals (Loughborough, UK)

Gas chromatography–mass spectrometry analysis

Sample preparation. All samples were randomized before sample preparation. PD fluid was thawed on ice and vortexed mixed for 15 s to ensure homogeneity. Aliquots of samples were spiked with 0.12 mg.mL^{-1} succinate d_4 acid in water and were lyophilized using standard procedures and samples were stored at 4°C until analysis.

Chemical derivatization. Chemical derivatization was performed as previously described [17].

Gas chromatography–mass spectrometry analysis. Samples were analysed in a randomized order on a 6890N gas chromatography (GC) and 7693A autosampler (Agilent, Cheadle, UK) coupled to a Pegasus III electron-impact (EI) time-of-flight mass spectrometer (Leco, Stockport, UK). Data were acquired over the m/z range 45–600 at an acquisition rate of 10 Hz. Raw data were processed in ChromaTof v2.32 as described previously [18] and exported as .txt files for data analysis.

Quality control samples. Aliquots (50 μL) of all samples were pooled and vortex mixed to prepare a pooled quality control (QC) sample. Subaliquots were prepared as for other samples as described above. The first five injections on to the gas chromatography mass spectrometry (GC-MS) system were QC samples (to equilibrate the system) followed by a QC sample every fifth injection and two QC samples at the end of the analytical batch. The QC samples were used to perform quality assurance of all data on a univariate basis as described previously [17]. For each detected chromatographic peak, the relative standard deviation (RSD) for all QC samples from the fifth injection were calculated and data for peaks where the RSD <30% for that peak were passed forward for data analysis and peaks with RSD >30% were removed before data analysis.

Direct injection mass spectrometry analysis

Sample preparation. All samples were randomized before sample preparation. Samples were thawed on ice and 100 μL was transferred to a

1.5-mL microfuge tube. Methanol (100–300 μL) was added (to perform deproteinization and to increase electrospray ionization efficiency) followed by vortex mixing and centrifugation at 15 800 *g* for 15 min. The supernatant (100–250 μL) was transferred to high recovery LC vials.

Direct injection mass spectrometry analysis. All samples were randomized before analysis. Samples were analysed in direct injection mode without chromatographic separation. This involved injection of 10 μL sample aliquots into a liquid mobile phase of flow rate 200 $\mu L.min^{-1}$ and composed of 50/50 methanol/water employing a Waters ACQUITY system (Waters Ltd, Elstree, UK). Data were acquired for 60 s and pre-processing involved averaging of scans in the time range of 0.2–0.45 s followed by background subtraction (0.05–0.15 s). All m/z values were exported as a .txt file for data analysis. A function was written using the Matlab® scripting language (http://www.mathworks.com/) to generate a data matrix (m rows × n columns) of the peak response in both negative and positive ion mode for each peak (m/z) (rows) in all samples (columns). Only those peaks present in ≥50% of the samples were included for further data analysis.

QC samples. Aliquots (50 μL) of all samples were pooled and vortex mixed to prepare a pooled QC sample. Subaliquots were prepared as for other samples as described above. The first 10 injections on the MS system were QC samples (to equilibrate the system) followed by a QC sample every fifth injection and two QC samples at the end of the analytical batch. The QC samples were used to assess the quality of data in the EPS study.

Data analysis

All the data were pre-processed prior to univariate and multivariate analysis. For the GC–MS data, the data were normalized to the internal standard (succinic acid d_4). The direct injection mass spectrometry (DIMS) data were normalized to the total response for each sample. Within Matlab®, exploratory multivariate analysis was performed using principal components analysis (PCA), an unsupervised approach which transforms a large set of related variables into a new, smaller set of independent variables, termed principal components (PCs). Each PC represents an axis in multidimensional space and corresponds to the direction of maximum variation of the original data. PCA was performed on data normalized to zero mean and unit variance, so that results were not dominated by a small number of high intensity peaks but gave equal weighting to all peaks. Univariate analysis was performed using the Wilcoxon signed-rank test to determine statistically significant differences between matched pairs of case and controls (earliest collected sample) with the critical P-value for significance set to 0.05. This test makes no assumption about a normal distribution of values and differences between the paired or matched data values are used to test for a difference between the two populations.

Metabolite identification

The putative or definitive identification of metabolic features detected on the GC–ToF-MS platform was performed by the comparison of retention index and EI mass spectrum to those recorded for authentic chemical standards and present in in-house libraries (e.g. Golm metabolome database or MMD in-house library) or in commercially available mass spectral libraries (e.g. NIST/EPA/NIH05 libraries). A definitive identification was assigned if the retention time (±10) and mass spectrum (match > 70%) were within the defined ranges. A putative identification was assigned if a match to a mass spectrum only was observed.

The putative identification of metabolic features detected on the DIMS platform was performed applying accurate mass data and the PUTMEDID-LCMS set of workflows (Brown M, Wedge D, Dunn WB, unpublished data). As different metabolites can be detected with the same accurate m/z (for example, isomers with the same molecular formula), multiple identifications can be observed for a single metabolic feature. A single metabolite can be detected as multiple features, each as a different type of ion (for example, protonated and sodiated ions).

Results

Validation of metabolomics methodology using GC–MS

Methods for mass spectrometric analysis have been developed and validated and have included the use of QC samples for quality assurance procedures [17, 19]. We wished to further develop these validated analytical methods to the analysis of PD fluids. Two experimental variables were assessed (i) lyophilized sample volume for reproducible detection of a high number of metabolites by GC–MS and (ii) the PD period (4 or 24 h) for reproducible detection of a high number of metabolites by GC–MS.

Lyophilized sample volume. The volume of sample lyophilized can be expected to influence the number of detected metabolites and the technical precision of the analytical method. The fluids applied in PD contain millimolar (mM) glucose concentrations which the authors perceived to be a potential problem. Typical single ion chromatograms (m/z 73) for the different dialysis fluids are shown in Figure 1. m/z 73 was chosen as this single m/z provides visualization of all metabolites in the sample as an artefact of the chemical derivatization process. The metabolites detected in the PD fluid after dialysis are shown in Table 1 and include sugars (mono- and disaccharides),

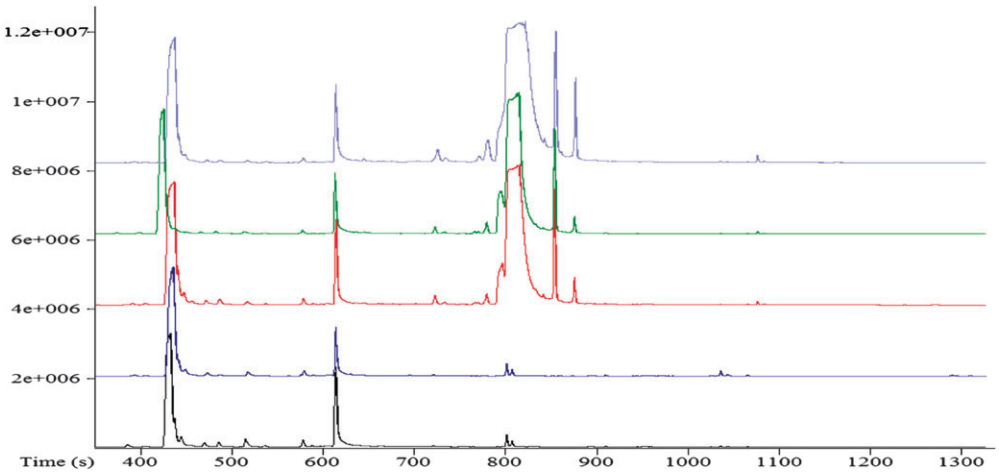

Fig. 1. GC–MS single ion chromatograms (m/z 73) for Dianeal 1.36 (red), Dianeal 2.27 (green), Dianeal 3.86 (light blue), Physioneal (black) and Extraneal (dark blue). The large peak with an approximate retention time of 800 s is identified as glucose. Samples were analysed using GC–MS method (a). The other major component was lactate (retention time of 430 s). The peak at 610 s is the internal standard (succinic d_4 acid).

sugar alcohols, amino acids, organic acids and other miscellaneous metabolites. All metabolites were definitively identified by matching to the retention index, and EI mass spectrum to that of an authentic chemical standard analysed under identical analytical conditions. Small subsets of these were detected in the dialysis fluid before PD and are therefore not necessarily derived from mammalian tissue only.

A study was performed to investigate the optimal sample volume (with respect to number of metabolite peaks reproducibly detected) to lyophilize. Aliquots (50, 100, 200, 400 and 800 μL) of a pooled QC sample were lyophilized. Incomplete lyophilization was observed for sample volumes of 400 and 800 μL. This result is most probably caused by the high residue mass not allowing complete dehydration.

The single ion chromatograms for m/z 73 are shown in Figure 2 for sample volumes of 100 and 200 μL. The peak with the high intensity and wide peak width at a retention time (*x*-axis) of 1000 s relates to glucose in the original PD fluid before dialysis. The Dianeal PD fluid was employed for all samples pooled in the QC sample. A 100% increase in sample volume did not result in a 100% increase in chromatographic peak area. This is a potential result of incomplete chemical derivatization at higher sample volumes because of a larger dried residue mass.

Further research assessed the reproducibility of data collected for multiple technical replicates of the QC sample. The technical precision associated with multiple injections of the same chemically derivatized samples was equivalent for both sample volumes. However, the technical precision for the analysis of multiple aliquots of the same sample passed through the sample preparation, chemical derivatization and analysis workflow was lower for sample volumes of

Table 1. List of metabolites definitively identified in GC–MS analysis of a pooled QC sample

Sugars and alcohols	Amino acids and derivatives	Organic acids and derivatives	Others
1,6-Anhydroglucose[a]	3,4-Dihydroxyphenylalanine	1-Octadecanol	1,3-Propanediol
2-Deoxyribose	3-Nitrotyrosine	2-(4-Hydroxyphenyl)ethanol	3,4-Dihydroxyphenylglycol
Arabinose	4-Hydroxyproline	2,3-Dimethylsuccinic acid	Guanine
Cellobiose	Alanine	2-Hydroxy-3-methylbutyric acid	Guanosine
Erythritol	Asparagine	3-Hydroxybutyric acid	Hexadecanoic acid[a]
Fructose[a]	Aspartic acid	Fumaric acid	Homocysteic acid
Gluconic acid[a]	Glutamic acid	Isocitric acid	Oxypurinol
Glucose[a]	Glycine	Lactic acid[a]	Phenol
Glucuronic acid	Isoleucine	Oxalacetic acid	Phosphate[a]
Isomaltose[a]	Leucine	Oxalic acid	Pseudouridine
Lactose	Lysine[a]	Oxomalonic acid	Stearic acid
Maltose[a]	Methionine	Saccharic acid	Sulphate[a]
Mannitol	*N*-Methyl proline	Shikimic acid[a]	Trimethylamine-*N*-oxide[a]
Mannose	Ornithine	Succinic acid[a]	Tyramine
Myo-inositol[a]	Phenylalanine	Tartaric acid	
Sedoheptulose[a]	Proline	Valeric acid	
Sorbitol	Serine		
Sorbose	Threonine		
Sucrose	Valine		
Threitol			
Threonic acid[a]			
Trehalose			

[a]Marks those detected in the dialysis fluid before PD.

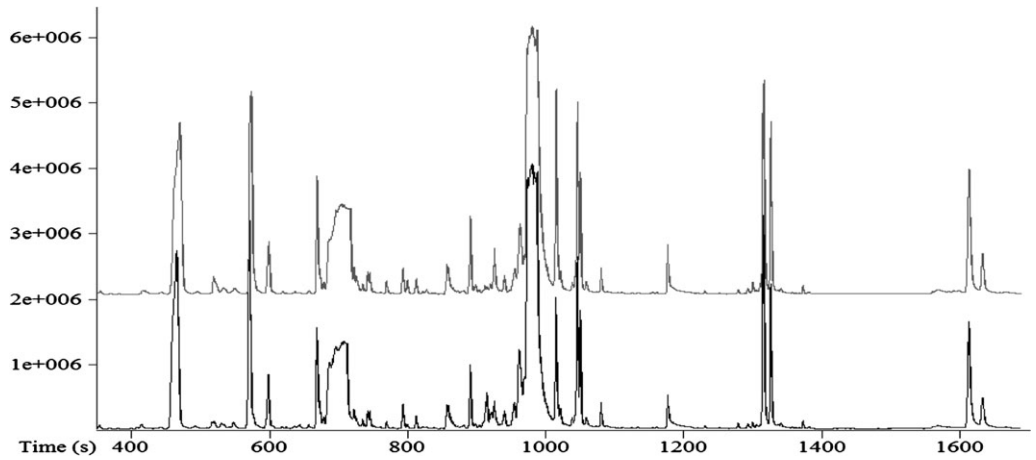

Fig. 2. GC–MS single ion chromatograms (m/z 73) for 100 μL (lower line) and 200 μL (upper Line) sample volumes of a pooled QC sample. Samples were analysed using GC–MS method (b).

200 μL rather than 100 μL. The number of metabolites detected for each sample volume was equivalent (78 versus 75 for 100 and 200 μL, respectively). Therefore, a sample volume of 100 μL was employed for future research as this provided equivalent numbers of detected metabolite peaks but with higher technical precision.

PD period sample collection. PD fluid is normally collected for 4 or 24 h during the PD period. It would be expected that the metabolic profile (or footprint) being analysed would contain a greater number of metabolites or metabolites at a higher concentration after 24 h. The samples showed a greater number of detected metabolites compared to the unused dialysate at both 4 and 24 h. Similar numbers of detected metabolites were observed for 4 and 24 h samples although the concentration of these metabolites can increase or decrease between 4 and 24 h. Analysis of 4 or 24 h samples is appropriate to provide 'fit-for-purpose' technical variation and a large number of detected metabolite peaks.

Validation of metabolomics methodology using DIMS

Sample preparation. The efficiency of ionization in electrospray sources is influenced by the solution composition and it is known that a composite of methanol and water is optimal for efficient ionization through ion production and droplet desolvation. Results show that addition of 200–300 μL of methanol produced similar numbers of detected ions, between 500 and 550 ions combined in positive and negative ion modes. Dilution in 100 μL methanol produced a lower number of detected ions (346), potentially caused by a lower efficiency of droplet desolvation in the electrospray source for the higher solution water content. It was decided that a dilution of 100 μL of sample in 200 μL methanol would be appropriate in these studies.

Other considerations. The mobile phase flow rate and composition were assessed. A flow rate of 200 $\mu L.min^{-1}$ produced a peak width of 40 s and an injection-to-injection time of 75 s. This was applied in all future work. A typical DIMS mass spectrum acquired in positive ion mode is shown in Figure 3. The PD fluid was composed of low–molecular-weight metabolites. However, the mass spectra are complex showing a wide range of detected metabolites.

Determination of metabolic differences in PD effluent related to EPS

Clinical characteristics and sample availability. The demographics for EPS and controls are shown in Table 2.

At the time of the first sample collection, the EPS group had been on PD for longer but this was not significantly different from the controls ($P < 0.05$). There was some variation in the PD fluids prescribed to these patients but again not significantly different between cases and controls. There were no significant differences in peritoneal *Kt*/*V* or peritoneal creatinine clearance (CrCl) between groups (Table 1). PET tests were performed at the time of sample collection and D/P creatinine levels showed no significant differences between the two groups. There were differences, however, in the renal *Kt*/*V* and renal CrCl parameters in that the group who subsequently developed EPS had lower mean values than the controls and a higher percentage were anuric, which has been associated with poor outcome *per se* and may not be specific to patients developing EPS. The mean time between the sample

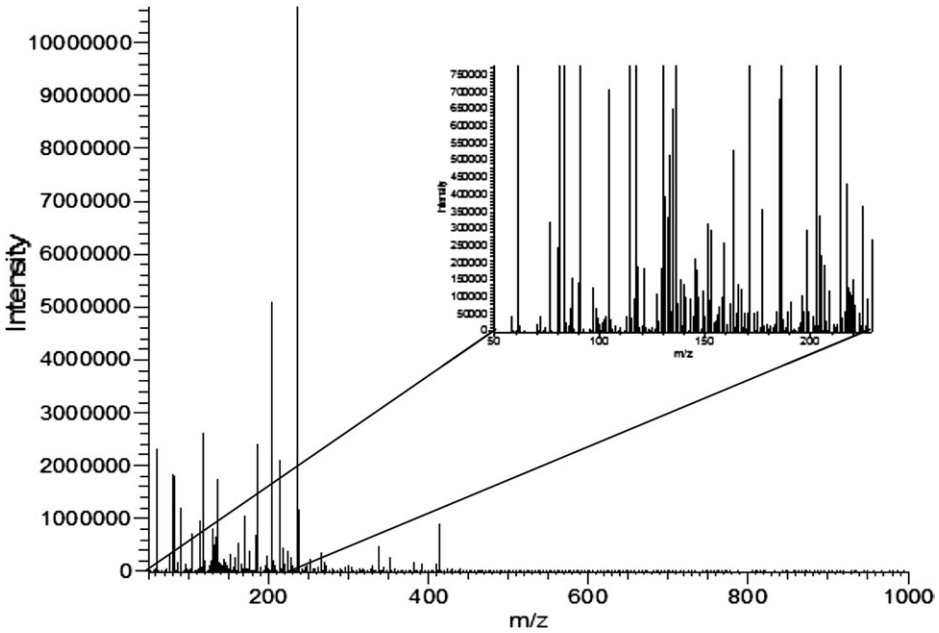

Fig. 3. Typical DIMS electrospray mass spectra for a QC sample acquired in positive ion modes. The mass range of 50–200 is shown in an expanded view to define the complexity of the data.

Table 2. Clinical characteristics of patients, EPS and controls[a]

	EPS case ($N = 11$)	Control ($N = 11$)	P-value
Age mean (IQR)	48 (39–56)	45 (35–59)	0.60
Gender F:M	5:6	6:5	NS
BMI mean (IQR)	25 (22–28)	26 (23–28)	0.78
Time on PD at sample date	1045 ± 368.6	638.3 ± 192.5	0.35
PD fluid (icodextrin)	7 (64%)	10 (91%)	0.30
PD fluid (biocompatible)	4 (36%)	4 (36%)	1.00
Total number of peritonitis median (range)	1 (0–6)	1 (0–7)	NS
D/P creatinine at sample date	0.82 ± 0.15	0.77 ± 0.11	0.29
Renal *Kt*/*V* mean (IQR)	0.285 (0–0.61)	1.007 (0.33–1.58)	0.02
Renal CrCl mean (IQR)	19.51 (0–33.2)	58.8 (19.3–88.2)	0.07
Peritoneal *Kt*/*V* mean (IQR)	1.75 (1.47–2.09)	1.602 (1.18–2.14)	0.48
Peritoneal CrCl mean (IQR)	63.57 (47.3–80.26)	51.44 (41.9–60.43)	0.10

[a]IQR, interquartile range.

collection and EPS diagnosis was 42.5 months (interquartile range 21.5–62.5).

Metabolic differences in PD effluent of case versus controls

Following the validation studies, an experiment was designed to assess the metabolic differences in PD effluent from patients later diagnosed with EPS compared to non-EPS diagnosed controls. Samples were prepared for GC–MS analysis using 100 μL of sample and for DIMS by dilution of 100 μL sample with 200 μL of methanol followed by centrifugation. All samples were analysed in a random order ensuring that no parameter directly correlated to analysis order. QC samples were included for quality assurance procedures as previously described [17, 19].

Univariate and multivariate data analysis was performed. Unsupervised multivariate PCA is applied to reduce the dimensionality of data while retaining data structure. Data for GC–MS and DIMS showed the separation of patient samples and dialysis fluid. However, after removal of data relating to fluid alone, there was no clear separation of case and control samples for GC–MS or DIMS data. However, the clustering of QC samples was tighter than patient samples, showing the variability of biological samples is greater than technical variability and provided confidence to proceed with univariate data analysis.

One hundred and twenty-four metabolite peaks present in the GC–MS data and 504 m/z ions (combined positive and negative ion modes) present in the DIMS data were used for univariate data analysis. Wilcoxon signed-rank tests were performed to determine statistically significant differences between the case and control populations. In GC–MS, 13 metabolite peaks were statistically different ($P < 0.05$) between cases and controls and 8 were chemically identified as shown in Table 3. In DIMS, 25 m/z peaks were statistically different ($P < 0.05$) between cases and controls and 19 were chemically identified as shown in Table 3. Metabolites detected applying GC–MS were definitively identified by matching retention index and mass spectrum to an authentic chemical standard. Metabolites detected applying DIMS were putatively identified and the accurate mass only was matched, via calculation of molecular formula(e), to a metabolite listed in the Manchester Metabolomics Database [20].

Discussion

The concept of applying 'metabolic profiles' in disease diagnosis originated in the 1940s with further developments in the 1960/1970s [21, 22]. However, considerable progress in metabolomics has been observed in the last decade after pioneering work by Fiehn [23] and Nicholson [24] applying mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, respectively. The scientific field of metabolomics, and the closely associated metabonomics, were conceived from these origins. Today, hypothesis-generating studies are routinely performed applying specific experimental workflows [15] to define new biological understanding in, among others, disease pathophysiology (e.g. insulin resistance [25]) and biomarkers (e.g. prostate cancer [26] and pre-eclampsia [27]). These studies require robust experimental design and data analysis and holistic and high-throughput analytical methods employing quality assurance procedures [17, 19]. Typically, NMR, MS (referred to as DIMS) or chromatography (GC, LC) coupled to MS platforms are applied in the detection of a wide array of metabolite classes, though no analytical platform can be used for the detection of all metabolites and therefore the application of complementary tools is appropriate [9].

The study presented here has shown the applicability of mass spectrometric-based metabolomics to the study of PD patients' effluent. The metabolic study of PD effluent has not been described previously though its collection is non-invasive and relatively easy as no metabolic quenching or specialized techniques are required as would be the case for serum, plasma and tissues. Methods for sample preparation and analytical methods have been developed and validated for GC–MS and DIMS applications. The dialysis effluent is highly appropriate for the study because its initial metabolic composition is simple and contains a limited number of metabolites (<20) and it is in direct contact of the site of action (peritoneal membrane) providing a sample sensitive to cell and tissue changes. GC–MS and DIMS were chosen

Table 3. Statistically significant metabolites ($P < 0.05$) observed when comparing EPS case versus control patients[a]

Metabolite	Analytical platform	P-value	Relative difference (median case/median control)
Diethanolamine and/or 2-amino-2-methyl-1,3-propandiol and/or reduced threonine and/or butanoic acid and/or 2-methylpropanoate and/or hydroxybutyrate	DIMS (positive ion)	0.0039	1.31
Trimethylamine *N*-oxide and/or aminopropan-2-ol and/or 2-methoxyethylamine and/or *N*-monomethylethanolamine	DIMS (positive ion)	0.0078	1.64
Isoleucine	GC–MS	0.0078	1.26
Methionine	GC–MS	0.0078	1.44
Sugar	GC–MS	0.0078	1.31
Furoic acid and/or ethanolamine and/or oxoproline	DIMS (negative ion)	0.0117	1.40
Phenylalanine	GC–MS	0.0117	1.55
Sugar	GC–MS	0.0117	1.56
Beta-alanine and/or alanine and/or sarcosine	DIMS (positive ion)	0.0273	1.31
Orthophosphate	DIMS (positive ion)	0.0273	1.32
2-Methylbutyl acetate and/or isoamyl acetate and/or ethyl isovalerate and/or heptanoic acid and/or methylhexanoic acid and/or dimethylpentanoic acid	DIMS (positive ion)	0.0273	1.26
Trehalose	GC–MS	0.0273	1.66
Thio-beta-D-glucopyranose and/or 5-amino-3-methyl-pyrrolidine-2-carboxylic acid	DIMS (positive ion)	0.0312	0.84
Sedoheptulose and/or glycero-D-manno-heptopyranose	DIMS (negative ion)	0.0312	1.49
1,3-Butadiene and/or 3-buten-1-amine	DIMS (positive ion)	0.0391	1.05
Glyceraldehyde and/or lactate and/or hydroxypropanoate	DIMS (positive ion)	0.0391	1.27
2-Hydroxymuconate semialdehyde and/or cis,cis-muconate and/or 2-oxo-2,3-dihydrofuran-5-acetate and/or cis,trans-hexadienedioate	DIMS (positive ion)	0.0391	0.90
Indoleacrylic acid and/or 2-(trimethylammonium)ethyl thiol and/or 4-(2-thienyl)butyric acid	DIMS (positive ion)	0.0391	1.26
Decenedioic acid and/or pseudouridine and/or uridine and/or ethyl-3-hydroxyhexanoate and/or hydroxyoctanoic acid	DIMS (positive ion)	0.0391	2.42
Pyromellitic acid and/or 4-guanidinobutanoate and/or 8-hydroxy-7-methylguanine	DIMS (positive ion)	0.0391	0.97
Galactosylhydroxylysine and/or lactose and/or maltose and/or nicotine glucuronide and/or epoxy-trimethyltrideca-2,6-dienoic acid and/or hydroxy-hexadecatrienoate	DIMS (positive ion)	0.0391	0.75
Erythronic acid and/or threonic acid and/or Hypoxanthine and/or 8-hydroxypurine and/or methyluric acid and/or oxaloglutarate	DIMS (negative ion)	0.0391	1.25
Leucine	GC–MS	0.0391	1.48
Tyrosine	GC–MS	0.0391	1.29
Lactose	GC–MS	0.0391	2.19

[a]The data are described in order of decreasing P-value. The fold difference (median peak area case/median peak area control) is shown. Data are only shown for identified metabolites, all unidentified metabolites have been removed.

as they provide complementary metabolic profiles through the detection of different metabolite classes [for example, GC–MS is applied in the detection of low-molecular-weight metabolites (e.g. amino acids and organic acids), whereas DIMS and the associated LC–MS are applied to the detection of higher molecular weight metabolites]. The complementary detection provided by these analytical platforms has been described previously [9]. DIMS was chosen instead of LC–MS because no lipids were detected in the initial studies and therefore the use of reversed-phase chromatography for separation of the low–molecular-weight polar metabolites would be limited as the chromatographic retention of these metabolites is low. If lipids were present, LC–MS would have been applied. It should be noted that DIMS is limited in its capabilities to identify metabolites and further targeted work to provide definitive identification of metabolites will be required in this and future studies.

The developed metabolomic methods have been applied to define metabolic differences in patients who subsequently developed EPS but in samples collected >3 years before diagnosis of EPS and before symptoms were clinically evident. These metabolic changes are likely related to the early onset of EPS (or EPS susceptibility) and were observed long before any symptoms and eventual diagnosis. As many identifications are defined as putative, groups of metabolites related by chemical similarity or metabolic pathways provide greater confidence in their accuracy compared to single metabolites. As this study is based on 11 controls versus 11 subjects and a critical P-value <0.05 with no multiple testing correction was applied, the grouping of metabolites provides greater confidence of a real effect being observed.

Changes in four classes of metabolites were observed; amino acids (six), ethanolamines (three), sugars (seven) and short-chain organic acids (eight). A recent publication assessing metabolic changes in serum of patients with PD showed changes in six amino acids including alanine, phenylalanine and methionine [28]. Changes in the concentrations of two amines were also observed and of these, trimethylamine-*N*-oxide was also observed to increase in the EPS patients

of this study. To an extent, our findings validate these results. However, the biological interpretation is limited and should be assessed with caution in view of the small sample size and potential confounding factors.

Six amino acids were observed at higher relative concentrations in PD effluent of EPS subjects compared to the controls. These were the branched-chain amino acids leucine and isoleucine, the aromatic amino acids phenylalanine and tyrosine along with beta-alanine/alanine and methionine. Increased concentrations of amino acids can be observed during cell damage through release of free amino acids or by proteolysis. In this respect, rat mesothelial cells have been shown to produce amino acids when cultured with hypertonic medium [29]. The observation that amino acids are increased in the peritoneum of EPS patients could be linked with the finding that dialysate IL-6 levels were higher after applying an amino acid-containing dialysis solution [30]. Amino acids may play a molecular mechanistic role in cell or tissue damage detected through increased IL-6 levels. The high amino acids concentrations observed in the EPS subjects, none of whom were exposed to amino acid supplemented solutions, appear to suggest that the use of these in dialysis solutions may in fact cause detrimental rather than protective effects on peritoneal tissue [31, 32]. These potentially detrimental effects, however, may be related to specific classes of amino acids different from those used in currently available dialysis solutions or in fact elevation of these levels might be indicators of the activation of other pathophysiological processes in these patients.

The role of primary amines and alcohols in patients on PD has been previously reported [28]. Two metabolites related to this metabolite class were elevated in EPS patients, diethanolamine and ethanolamine. These metabolites are precursors in glycerophospholipid biosynthesis, related to construction of cellular membranes. Trimethylamine-*N*-oxide was also found at higher concentrations in the EPS subjects. These three metabolites have been described as organic osmolytes in human cells and tissues to provide effective roles in cell preservation in water and salt stressed systems [33]. These may be produced at higher concentrations in EPS patients or cell damage results in increased release into the dialysate. These metabolites are also uraemic toxins and may be expected to be elevated in patients on dialysis and in renal failure especially those with less residual renal function. This is important in that the controls had a higher mean creatinine clearance compared to the EPS group although not statistically significant. The finding that anuria was more common among EPS patients is not surprising since it is a recognized risk factor for this condition, probably due to the association between anuria and time on PD as well as increased exposure to glucose based dialysate in such patients. However, it does pose a potential confounder since anuria also would lead to a significant alteration of the molecular profile. This does not negate the importance of our findings since this may provide a potential mechanistic link between anuria and the development of EPS.

The disaccharides lactose and trehalose were observed at higher concentrations in EPS subjects and may also play a protective role as an osmolyte. Organic acids in plasma have been reported to be higher in progression to chronic renal failure [34]. The higher organic acid concentrations observed in the study reported here could be caused by membrane damage and increased leakage of these into the PD effluent.

Ascorbate-6-phosphate was observed to be at lower concentrations in EPS patients. This is derived from ascorbate, an antioxidant. Reduced levels of ascorbate have been identified in uraemic patients and patients on haemodialysis [35, 36]. Ascorbate represents one of the most prominent antioxidants both in plasma as well as intracellular, exerting beneficial effects by inhibition of lipid peroxidation and by reducing endothelial dysfunction. Inflammation and reactive oxygen species (ROS) production are associated with EPS and a reduced level of ascorbate-6-phosphate may be related to an increased use of ascorbate for removal of ROS. It may also imply that dietary intake or absorption of ascorbate may be reduced before the onset of EPS. A prospective study is needed to determine whether PD patients with very low ascorbate levels are at risk of EPS and hence whether supplementation could be beneficial.

We fully acknowledge that this study has a number of limitations. The prevalence of EPS (1.8% in the UK arm of the EPS fluid global study; 600 subjects recruited over a 6-year period) limits the number of samples available and therefore studying large numbers of cases is difficult. However, in this proof-of-principle study, we have provided pointers for further research in a discovery phase investigation. Further validation studies are required to overcome potential confounding factors and the small sample size, using independent sample sets, to further test the hypotheses constructed (see [13] for further information). Closely matched controls were not available and potential confounding factors are present (e.g. renal Kt/V and renal CrCl) which can be expected to influence the metabolic profiles determined and these require further investigation in larger studies. The time on PD is also higher for the EPS subjects compared to controls (although this was not statistically significant at $P < 0.05$). The time on PD relates to the number of days on therapy prior to diagnosis and this was the length of time used to match the controls. The sample date was prior to diagnosis as by the time this was made, patients had stopped PD. This factor makes it difficult to look at effluent markers at the time of EPS diagnosis and highlights the importance of looking for early markers.

These confounders limit the biological conclusions that can be drawn from the data. It may be expected that differences in residual renal function will create higher osmotic gradients which would influence the rate of transport into the EPS fluid and the concentration of metabolites in the EPS fluid. These can be classed as uraemic toxins. Further analysis of the data shows that many of the metabolites discussed show no correlation between metabolite concentration and these confounding factors, indicating that the confounding factors do not have an influence on the concentrations of these metabolites.

In summary, this proof-of-principle study has highlighted inherent differences in the metabolic profiles of patients who subsequently developed EPS compared to matched controls and that these changes were detectable long before diagnosis. The causes of these changes in the metabolic profile require further investigation to ascertain whether these are based on pathological processes related

to EPS or the effect of differences in residual renal function between case and control groups. However, the results appear to imply that subtle long-term alterations may be occurring in the peritoneum driven by or observed in metabolic networks. The observation of groups of metabolites of similar chemical structure or function provides increased confidence to the results. Collection of larger prospective cohorts or the integration of multiple international cohorts would provide a larger sample size for EPS patients.

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