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Journal:	*Kidney International*
Manuscript ID	KI-11-16-1766.R1
Article Type:	REVIEW
Date Submitted by the Author:	n/a
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Keywords:	peritoneal dialysis, peritoneal membrane, fibrosis, inflammation, peritonitis
Subject Area:	Dialysis

Biomarker Research to Improve Clinical Outcomes in Peritoneal Dialysis – Consensus of the European Training and Research in Peritoneal Dialysis (EuTRiPD) Network

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Running head: Biomarkers in PD effluent

Abstract

Peritoneal dialysis therapy (PD) has a substantial need for biomarker as tools to identify patients that are at highest risk for PD-related complications and to guide personalised interventions that may improve clinical outcome in the individual patient. In this consensus paper, members of the European Training and Research in Peritoneal Dialysis Network (EuTRiPD) review the current status of biomarker research in PD and suggest a selection of biomarkers that might become relevant for the care of PD patients and which is directly accessible in PD effluents.

Currently used biomarkers collected in a Delphi procedure were first triaged for inclusion as surrogate endpoints for a clinical trial (IL-6, IL-8, *ex-vivo* stimulated IL-6 release, CA-125, AOPP). Next, novel biomarkers were selected as promising candidates for proof-of-concept studies, and differentiated into inflammation-signature (including IL-17, M_1/M_2, T_{reg}/Th17), MMT-signature (including miR-21, miR-31) as well as signatures for senescence and inadequate cellular stress responses. Finally, the need to define pathogen-specific immune fingerprints and phenotype associated molecular signatures (PAMS) utilizing effluents from clinical cohorts of PD patients, and 'omics technologies and bioinformatics/biostatistics was expressed as need for future joint research efforts.

Biomarker research in PD offers the potential to develop valuable tools to improve patient management. However, for all biomarkers discussed in this consensus paper, the association of biological rationales to relevant clinical outcomes remains to be rigorously validated in adequately powered, prospective independent clinical studies.

Keywords: renal replacement therapy, surrogate markers, peritonitis, epithelial–mesenchymal transition, ultrafiltration failure, proteomics

Background

Peritoneal dialysis (PD) is an effective, home-based form of renal replacement therapy that promotes patient autonomy. A significant proportion of patients who initiate PD suffer from PD-related clinical complications that may limit duration of treatment, including peritonitis and peritoneal membrane damage.[1] PD patients are also at high risk of other serious and life threatening illnesses, most notably cardiovascular diseases. Current approaches to patient monitoring, however, are mostly limited to approximating delivered dose of dialysis and measurements of membrane transport status. Consequently, despite considerable improvements in patient management and overall technique survival, there is a substantial unmet medical need for biomarkers as tools to identify patients that are at the highest risk and to guide personalised interventions in order to improve clinical outcome of PD in the individual patient.

In the clinical context, a biomarker is a proxy of disease mechanisms, which gives relevant information for decision-making regarding the diagnosis and/or therapy of a patient. Another classical definition is: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention".[2] This information may become directly obvious from the molecular processes that reflect disease status, such as increased levels of inflammatory mediators in biological fluids due to increased production and release from inflamed tissues or local leukocytes. However, biomarker levels may also reflect mere changes of distribution between compartments, such as leakage from intracellular into extracellular, or spill over from systemic into local compartments by altered clearance.[3] Accordingly, identification and interpretation of appropriate biomarkers is not trivial, and the clinical value of attractive biomarker candidates is difficult to predict and requires careful preclinical and clinical validation.[4]

This consensus paper focuses on those biomarkers that are thought to be relevant for the care of PD patients, but are limited to the "local" peritoneal level, *i.e.,* biomarkers that are directly

accessible in PD effluents. In what clinical circumstances would these biomarkers be of benefit? Used as a risk-assessment tool (see figure 1), prognostic peritoneal biomarkers might help to identify patients that are at highest risk for PD-related complications. For example, biomarkers that reflect chronicity of peritoneal inflammatory processes might identify patients prone to progressive loss of membrane function. Similar to sepsis research, biomarkers that reflect depressed immuno-competence might identify increased infectious susceptibility in PD, such as PD-related peritonitis. Monitoring a set of biomarkers that reflects the activity of relevant pathomechanisms might thus help to guide therapeutic decisions or, following therapeutic interventions, allow early discrimination between responder and non-responder subgroups. Introduction of such predictive biomarkers (see figure 1) will likely facilitate the implementation of stratified medicine into the clinical setting of PD. For example, a high pro-inflammatory status in a given PD patient might necessitate the introduction of anti-inflammatory local therapy by novel PD fluids. However, biomarkers predicting a particularly high risk for PD-related complications might also allow a timely switch to alternate forms of renal replacement in non-responding patients. Importantly, combinations of these biomarkers may also be used as surrogate parameters for well-defined hard outcomes in the clinical development for novel PD fluids. Such biomarkers are particularly relevant tools as the “hard outcomes” require large studies with several hundred patients observed over several years and thus present major logistic and economic obstacles for dearly needed early clinical trials in PD.[1] Finally, biomarkers might also be implemented as a diagnostic tool. For example, a certain pattern of cytokines might reflect the nature of the causative pathogen in peritoneal infection and thus allow gaining quick and reliable diagnostic information relevant for therapeutic decisions (e.g., the guidance of antibiotic regime).

FIGURE 1

TABLE 1

Several effluent biomarkers, such as CA-125 (believed to represent mesothelial cell mass) and IL-6 (indicating local inflammation), have already been shown to be informative at the population level in the follow-up of PD patients. However, at present the integration of effluent biomarkers into clinical decision making in PD is only modest.[5] In this consensus paper, members of the European Training and Research in PD Network (EuTRiPD) discuss the current status and prospect of a selection of novel effluent biomarkers.

Hypothesis-Driven Peritoneal Biomarker Research

The recent development of effluent biomarkers in PD has predominantly been hypothesis-driven and based on pathologies and pathomechanisms found to be relevant for the course of disease in PD. The biomarkers discussed in the following section are primarily related to chronic inflammation, peritoneal membrane remodeling and peritoneal infection.

Biomarker research in the context of Chronic Peritoneal Inflammation

Although not yet used in clinical routine, markers of chronic inflammation should be predictive of reduced survival in PD and HD patients, however, use of inflammatory cytokines and other related molecules as biomarkers must take into account of the complexity of their function and associations in this context. The most thoroughly studied marker of inflammation in PD patients is Interleukin-6 (IL-6), which is also a major target for therapy in other diseases.[6] Systemic levels of IL-6 and its soluble receptor are elevated in patients with end-stage renal disease (ESRD), and circulating IL-6 levels at initiation of PD therapy predict the mortality risk.[7] Multiple factors may contribute to circulating IL-6 levels, including persistent or episodic bouts of infection-inflammation, obesity, and metabolic alterations. Some of this excessive risk also appears to be genetically controlled, with polymorphisms in the IL-6 gene being associated with a high IL-6 producer status and reduced survival rates in patients undergoing PD. Impaired clearance of IL-6 in patients with severely diminished kidney function may also be contributing.

Cellular composition of the peritoneal effluent offers a unique view on the tissue-resident immune system, and effluent cell and cytokine profiles reflect complex immunological interactions active in the peritoneal membrane and cavity in response to chronic PD fluid exposure and intercurrent infections. With regards to its role as a peritoneal biomarker, IL-6 is increased in the effluent of patients suffering from acute bacterial peritonitis, where IL-6 is required for effective bacterial clearance in the acute response to infection.[8] Moreover, elevated levels of pro-inflammatory cytokines might also be indicative of sub-clinical, smoldering infections such as bacterial biofilms on PD catheters.[9] Importantly, experimental evidence in animal models links persistent peritoneal IL-6 generation to membrane change/fibrosis[10] and angiogenesis.[11] These processes may well bear clinical relevance as is suggested by the results of the Global Fluid Study, a multinational, multicenter, prospective, cohort study in 959 PD patients with up to 8 years of follow-up. Here it was found that local peritoneal and systemic inflammation are uncoupled, and that local, not systemic, inflammation is a main determinant of changes in peritoneal small solute transport rate that is observed over time.[12]

Recently, T helper (Th)-17-mediated inflammatory response, and in particular the cytokine IL-17, have been shown to play a central role in peritoneal damage.[13,14] Experimental modulation of the Th17 response and/or enhancing the regulatory T cell (Treg) response may preserve membrane function.[15-17] Thus, chronic inflammatory damage of the peritoneal membrane can be modulated, at least in part, through regulation of the Th17/Treg balance.[18] Other cytokines are linked to specific PD-associated patient subgroups and characteristics. The chemokine CCL18 (also known as PARC, DC-CK1 and MIP-4) had originally been described to be predictive of encapsulating peritoneal sclerosis (EPS), but this finding was not confirmed by recent studies.[19,20] Peritoneal levels of CXCL8 (IL-8) were measured as part of the immune fingerprinting during bacterial peritonitis[21], yet increased secretion of IL-8 may also be a feature of the senescence phenotype (see below). Thus, high readings of inflammatory markers such as IL-6 or other cytokines are currently suggested as emerging biomarkers for

further clinical development (see table 1) but may require differential interpretations depending on the clinical context.[12,22-29]

Biomarker research in the context of Peritoneal Membrane Remodeling

Currently, data on peritoneal transport characteristics from the peritoneal equilibration test (PET), and in particular their change over time, are used to guide PD patient treatment and management. The previously well-discussed candidate biomarker CA-125 was rapidly applied to estimate mesothelial cell mass as a surrogate parameter for the peritoneal membrane status in studies comparing different dialysis fluids (see table 1), although its utility in this context remains contentious.[3,5,30]

Peritoneal mesothelial-to-mesenchymal transition (MMT) and inflammation establish a feedback loop in which the MMT process may induce inflammatory mediators, which again promote MMT.[31] Moreover, fibroblast play a role in this process.[32] As a result the peritoneal membrane undergoes a progressive remodeling with the accumulation of extracellular matrix and fibrosis. Peritoneal infections aggravate the peritoneal membrane remodeling process. Patients who remain infection-free, however, also evidence PD induced inflammation and fibrosis, which may result in loss of peritoneal membrane function and ultimately cessation of PD.[33,34] Assessment of the progressive morphological alterations would require repetitive peritoneal biopsies, which is, however, feasible in clinical routine only at time of catheter insertion and at time of subsequent abdominal surgery.[35] Alternatively, the *ex vivo* study of effluent-derived mesothelial cells might be useful to monitor peritoneal remodeling.[36] Effluent mesothelial cells show a progressive loss of epithelial phenotype and acquire fibroblastic characteristics through MMT.[37-39] MMT is a complex process during which mesothelial cells are transformed into fibroblast-like cells with the capacity of producing a wide spectrum of inflammation, fibrosis and angiogenesis mediators.[33] The *ex vivo* expression of molecules associated with MMT in effluent mesothelial cells is associated with peritoneal transport status.[40,41] Moreover, levels of MMT-associated molecules in the PD effluent, including VEGF,

CTGF/CCN2 and Gremlin-1, were also found to correlate with peritoneal transport.[42-44] The next goal is to develop a combination of MMT-related molecules that can be measured in the PD effluent at once (MMT-Chip) and offer diagnostic/prognostic value.

Another line of research is the identification of different peritoneal fibroblast phenotypes whose features may serve as a fingerprint of changes in the peritoneal membrane during PD. Activated fibroblasts (myofibroblasts) originate from several precursors, including mesothelial cells, resident fibroblasts, endothelial cells, and circulating fibrocytes and contribute most to tissue fibrosis.[45] Experiments using inducible genetic fate mapping indicated type I collagen–producing submesothelial fibroblasts as specific progenitors of α-SMA–positive myofibroblasts that accumulate progressively in animals exposed to sodium hypochlorite, hyperglycemic dialysis solutions, or TGF-β1, suggesting an alternative mechanism of peritoneal fibrosis to MMT.[46] Moreover, fibroblasts expressing Thy-1 (CD90) appear to have an increased ability to acquire a myofibroblastic phenotype.[32] Thus, the proportion of Thy-1^+ and Thy-1^- fibroblasts residing in the peritoneum may potentially identify individuals who are more prone to myofibroblast expansion following peritoneal injury.

Biomarker research in the context of Peritoneal Infection

Peritoneal infection remains one of the main culprits for technical failure and patient morbidity in PD. Whilst white cell counts (WCC) and the proportion of granulocytes in the peritoneal effluent are widely accepted as biomarkers for infection[47] less progress has been achieved in identifying biomarkers that discriminate between infection and non-infectious inflammation. Culture-based diagnosis of infection is slow and error-prone, with 20-25% of cultures remaining negative despite distinct clinical and biochemical signs of bacterial infection.[48] Direct identification of pathogens using state-of-the-art technologies such as PCR or mass spectrometry with satisfactory sensitivity and specificity remains a challenge.[49] Culture-negative episodes of peritonitis may include cases of sterile inflammation that may not require antimicrobial therapy but are often masked by inappropriate sample processing or

culture/detection conditions, especially for fastidious organisms and viruses.[50] When accurate microbiological diagnosis in patients presenting with acute symptoms is not possible, peritonitis management is largely empirical, and treatment with broad spectrum antibiotics is recommended. Basic biomarkers identify culture-negative episodes, with culture-positive infections having greatly elevated WCC values and higher frequencies of granulocytes.[21] Moreover, levels of peritoneal effluent cytokines are lower in culture-negative episodes, with e.g., IL-1β and IL-10 showing potential for a distinction from infectious peritonitis.

When infection is present, biomarkers that are specifically associated with different types of pathogens, maybe useful to distinguish fungal, bacterial and viral infections and thus help inform the choice of treatment. In the absence of clear diagnostic parameters, prophylactic treatment of all peritonitis patients with antifungals has been recommended whenever a course of antibiotics is administered.[51] In the case of bacterial pathogens, early discrimination between Gram-positive and Gram-negative species would greatly reduce prescription of broad spectrum antibiotics, Gram stainings of patient samples are routinely performed but lack sensitivity.[52] The availability of pathogen-specific biomarker signatures, combined with the early identification of antimicrobial resistance patterns at the point of care, would represent a major breakthrough in the accurate diagnosis and targeted therapy of peritonitis.[53] For instance, biomarkers of particular relevance for the prediction of Gram-negative infections may include comparatively higher levels of the cytokines IL-1β, IL-10 and tumor necrosis factor (TNF)-α compared to Gram-positive infections, combined with larger numbers of infiltrating neutrophils and elevated frequencies of peritoneal γδ T cells.[21]

A biomarker predicting risk for infections might also help to guide clinical decisions to improve outcome in PD patients with compromised immunocompetence who are particularly susceptible for infectious complications.[54-57] In critically ill patients depressed lipopolysaccharide (LPS)-stimulated release of TNF-α from whole blood has successfully been used to detect systemic immunosuppression and to monitor immunomodulatory therapies.[58-60] In PD, several studies have shown that *ex-vivo* stimulation of peritoneal macrophages isolated from PD effluents results in increased cytokine release compared to constitutive

expression.[55,61-64] Typical stimuli of cytokine release from peritoneal leucocytes are LPS and Pam3Cys as ligands of Toll-like receptors (TLRs); typical read-outs are TNF-α and IL-6 as rapidly reacting cytokines of known clinical relevance. Such assays demonstrated an improvement of peritoneal macrophage function in patients dialyzed with neutral pH solutions compared to patients receiving conventional therapy.[55,61-63] *Ex-vivo* stimulated cytokine release in peritoneal effluents is therefore suggested as promising biomarker (see table 1), however, prospective studies in larger PD populations are dearly needed to validate these assays as surrogate parameters of immune competence and clinical outcome.

Finally, there is a need for biomarkers to reliably predict outcome of infectious complications of PD, to identify patients at risk of downstream complications, recurrent/relapsing infections and even death, where extended hospitalization may be appropriate and catheter removal be recommended. Moreover, infectious peritonitis might trigger smoldering sterile inflammation, a condition requiring timely diagnosis to interrupt the subsequent vicious cycle activating multiple of deleterious peritoneal pathomechanisms.[65] Different levels of local and/or systemic immunocompetence and types of pathogens carry individual risks and outcomes, and as such early biomarker-based stratification of patients may ultimately determine improved clinical outcomes from infection. In this context the identification of high white cell counts or elevated levels of peritoneal $V\delta2^+$ T cells as early predictors of subsequent technique failure are promising developments.[53,66]

Biomarker research in the context of further pathomechanisms relevant to PD

Cellular senescence is a complex biological program triggered by stimuli that can put the integrity of the genome at risk.[67] It is characterized by irreversible growth arrest, distorted cell morphology and altered cytokine secretion (including increased release of IL-6 and CXCL8).[68] Peritoneal mesothelial cells in mice exposed chronically to PD fluids exhibit a phenotype resembling that of senescent cells,[69] Likewise, the *in vitro* exposure of human peritoneal mesothelial cells to high concentrations of glucose results in accelerated development of the

senescent cell phenotype,[70] partly through increased oxidative stress.[71] Consequently, the appearance of senescent mesothelial cells in the peritoneum may be indicative of peritoneal membrane deterioration. However, the clinical exploitation of this concept is hampered by the absence of a universal senescence marker [72] and difficulties in detecting senescent mesothelial cells *in vivo*. In this respect, it might be easier to detect senescence among mesothelial cells shed to the peritoneal effluent.[73]

As oxidative stress is among the leading causes of premature senescence, the expression of oxidative stress biomarkers may be useful as surrogate indicators of peritoneal membrane deterioration. Oxidative stress is typically defined as a disturbance in the pro-oxidant / antioxidant balance in favor of the former.[74] Reliable markers of oxidative stress should consistently increase or decrease during periods of oxidative stress but not be impacted by other cellular processes.[75] However, the interpretation of changes in currently measured biomarkers of oxidative stress is challenging, as increased cellular antioxidant levels may either reflect an improved antioxidant status or a compensatory response to an oxidative insult. While several biomarkers of oxidative stress have been tested in PD patients such as advanced oxidized protein products (AOPP, see table 1) in peritoneal effluents[76-78], the comprehensive assessment of a broad panel, especially in the context of cellular senescence, remains to be performed and validated in clinical outcome related research.

The combination of these pathomechanisms may result in abnormal cellular stress responses, potentially hampering peritoneal repair and propagating chronic inflammation.[79,80] In experimental PD, cellular expression of the inducible 27 kDa and 70 kDa heat-shock proteins (Hsp27, Hsp72) has been shown to be dampened by PD fluid toxicity and inflammation.[80,81] In effluent derived mesothelial cell cultures from PD patients, expression of Hsp27 and Hsp72 was demonstrated to be influenced by *in vivo* and *ex vivo* MMT processes.[82] Investigating the cellular stress responses at the proteome level (see also below) demonstrated an inadequate mesothelial Hsp72 expression that could be restored by therapeutic interventions.[83] These data suggest that the assessment of the adequacy of peritoneal cell stress responses might yield promising biomarkers to guide novel therapeutic interventions. To this end, alanyl-

glutamine dipeptide is currently tested in clinical trials and has been shown to restore adequate cellular stress responses (NCT01353638, EudraCT2013-000400-42).[84] This compound has recently been shown to increase the attachment of *N*-acetylglucosamine to proteins (*O*-GlcNAcylation) in mesothelial cells and improve the resistance against PD-fluid toxicity.[85] Accordingly, the monitoring of *O*-GlcNAc levels in peritoneal cells might evolve as an independent novel biomarker for the preservation of peritoneal health in PD.

A particularly interesting approach to establish and/or validate biomarkers is given in the pediatric PD population. Chronic renal failure is rare in this age group but in the majority of children based on congenital disorders that are mostly limited to the kidneys and the urinary tract, *i.e.* ruling out associated tissue alterations linked to systemic inflammation and aging. In a world-wide effort, the International Pediatric Peritoneal Biobank has already collected more than 300 parietal peritoneal and 200 omental tissue specimen, which allow for a systematic comparison of the peritoneal membrane whole genome and proteome expression pattern in health, uremia, and PD. Reference values of the healthy peritoneal membrane ultrastructure (0-60 years) reflect major age specific particularities and now provide a framework for future histomorphometric analyses and peritoneal transport modeling approaches[35]. Preliminary transcriptomics and proteomics findings obtained from omental arterioles, *i.e.* of tissue samples not directly exposed to PD fluid but giving insight into uremia and PD associated pathomechanisms of cardiovascular disease, elucidate the fundamental role of inflammatory pathways with distinct elements of the innate immune system being consistently upregulated on RNA and protein level.

FIGURE 2

Non-Hypothesis-Driven Peritoneal Biomarker Research (Open "Omics" Approach)

Biomarker research based on the alternative “open approach” is increasingly productive due to the improved availability of well-curated biobanks holding material from clinical cohorts, and due to increasing experience with the use of 'omics technologies and bioinformatics/biostatistics in the setting of PD.

So-called unbiased strategies for biomarker identification may overcome the limitations of candidates based on current hypotheses and available literature. Focusing on “known” pathomechanisms and measuring surrogates of these pathomechanisms are likely to produce more of the same information, and may thus overlook stronger predictors of outcome of disease and therapy.

‘Omics techniques aim to assess all biological molecules of a defined category (proteins, nucleic acids, lipids, carbohydrates) in a biological system at the same time, thereby producing a snapshot of the investigated sample. The result obtained from omics techniques is therefore a system-wide list of absolute or relative abundance values, where usually the number of individual features (proteins, transcripts, metabolites etc.) by far exceeds the number of samples measured. This undersampling leads to a number of challenges regarding the statistical exploration of the data, including the necessity to account for multiple testing of hypotheses.

Importantly, the coverage of all molecules of a given type allows building statistical models not only relying on a single marker but on a collection of markers, best discriminating between different clinical outcomes, a so-called molecular signature. As single biomarkers can hardly reflect the biological complexity of underlying diseases, comorbidities, genetic background and context-dependent biological responses, molecular signatures are usually able to outperform individual surrogate markers and therefore may represent more relevant biomarkers.

Proteomics in the context of PD

In the field of PD only a limited number of ‘omics studies has been performed, mostly trying to identify pathomechanisms *in vitro* or in animal models, or individual biomarkers in PD effluent. Using *in vitro* models, proteomics identified a molecular signature of the stress response to

PD-fluid exposure based on 60 mesothelial cell proteins, which were then used for evaluation of pharmacological interventions [83,86]. In animal models, a proteomic signature of extracellular matrix (ECM) proteins was employed to characterize the effect of specific gene deletion on MMT mechanisms.[87] None of these approaches have yet been applied to clinical samples.

PD effluent represents a particularly attractive material for biomarker research as it contains a rich pool of biomolecules that are indicative of the peritoneal transport status as well as of peritoneal health, ongoing pathological processes and even the status of underlying disease and comorbidities. However, only few studies previously have used proteomics to investigate PD effluent,[88-99] identifying a very limited portion of the peritoneal proteome. This is due to the fact that high abundance proteins originating from plasma mask low abundance proteins like cytokines and chemokines as well as proteins from cellular origin. When focusing on these low abundance biomarker candidates, depletion strategies are needed such as. affinity-chromatographic separation or semi-specific precipitation of proteins. A particularly promising approach is the application of bead-based depletion and enrichment techniques, such as the combinatorial peptide ligand library (CPLL) beads, also called equalizer beads. In this case a limited number of binding sites for all potential proteins is available on the surface of the beads, so that highly abundant protein species are saturated and the excess is removed during the washing steps, whereas low abundant proteins are relatively enriched and therefore detectable by analytical techniques such as 2D gel electrophoresis and/or liquid chromatography coupled mass spectrometry (LC-MS).[100]

Transcriptomics in the context of PD

PD effluent also contains a significant amount of nucleic acids, where messenger-RNA (mRNA) transcripts might represent information about the activation status of individual genes. Whilst free-floating mRNA in PD effluent is difficult to use as a biomarker due to its limited stability, more stable species such as microRNAs should be potentially detectable in PD effluent and used as biomarkers. MicroRNAs are short RNAs that bind to specific protein-coding messenger RNA targets and repress synthesis of their respective proteins. There are

more than 2000 microRNAs encoded in the human genome, each predicted to repress the synthesis of many proteins. MicroRNAs thus act as a complex layer of repressive regulation for protein synthesis, and are critical for normal development, physiological processes, and pathological mechanisms. Changes in microRNA expression are strongly linked to fibrogenesis in many contexts (reviewed in [101]).[102-104] In studies of their role in peritoneal dialysis to date, miRs -15a, -17, -21, -30, -192 and -377 were associated with peritoneal transport characteristics in a cohort of 110 PD patients (reviewed in [105]).[97,106-108]

Metabolomics in the context of PD

PD effluent comprises of a significant amount of biological metabolites as well as small molecules originating from the original PD fluid and potential derivates, such as glucose adducts or break-down products of polymer osmotic agents. This fact renders metabolomics a particularly attractive technique for generating molecular signatures from PD effluent. Up to now a limited number of studies has been carried out, mainly focusing on proof-of-feasibility. One study employed gas-chromatography coupled to MS or direct injection MS for generation of metabolomics profiles of PD effluent from patients who later developed EPS compared to matched controls.[109] One of the challenges in metabolomics analysis is the high redundancy of small molecule masses based only on their exact mass. Therefore in the second available study, a high resolution accurate mass (HR/AM) approach was combined with comparison of experimental and *in-silico* fragmentation of candidate molecules to increase the likelihood of unambiguous identification of metabolites increasing in PD effluent during a controlled 4 h PET dwell.[110]

Defining phenotype associated molecular signatures in PD

Currently only limited data from omics approaches using PD effluent as sample material is available. In the future, it would be particularly important to use biobanks generated from well-defined clinical cohorts in order to define molecular signatures reflecting the pro-inflammatory

phenotype and/or peritoneal membrane status as has been discussed above for the hypothesis-driven approach (see also Figure 1).

The amount of data generated from 'omics techniques can be enormous. Adequate IT infrastructure for storage and retrieval of the data as well as streamlined statistical methods are therefore a pre-requisite. The statistical analysis approach can either be "supervised", using perfectly described clinical cohorts, linking a clinical phenotype to a molecular signature, or "unsupervised", where algorithms for clustering the data or reducing its dimensions are employed to find subgroups of samples which then have to be interpreted on the basis of the available clinical data. Hierarchical clustering methods with its diverse range of distance measures influencing the character of the analysis, principal components analysis (PCA) where the first component explains the most variability in the data on the basis of the original variables (loadings) by an Eigen-vector projection of orthogonal dimensions are prominent examples of the unsupervised statistical analysis approach. Regarding the supervised approach, machine learning techniques, where a training set of samples is used to select, weight and combine classifiers which are then applied to a cohort of unknown cases/samples, might be of specific use in the future. However, until now the application of such high-end statistical approaches is still rare in the field of biomarker research, and even more so in the field of PD. Usually, standard test statistics are employed and at best, individual promising candidate markers are combined using linear models, which in many cases leads to overfitting of the training data, thereby limiting the clinical applicability later on.

The technical validation of molecular signatures with biomarker potential, if possible using orthogonal analytical techniques, as well as the verification of the predictive power of signature candidates in independent sample cohorts is crucial to ensure clinical value. Eventually, a molecular signature that is translated into clinical use will likely have to be measured using rapid and cost-efficient methods, such as clinical chemistry techniques or multiplex ELISA. For some applications targeted LC-MS methods might be available as a clinical routine tool in the near future, enabling clinical integration of molecular signatures.

FIGURE 3

Conclusion and Clinical Outlook

Biomarker research in PD offers the potential to develop valuable tools to monitor therapy and improve patient management. However, for all biomarkers discussed in this consensus paper the association of biological rationales to clinically relevant outcomes (= phenotypes) remain to be rigorously tested. Comparable to the development of a novel drug, once the biological relevance of a candidate biomarker is established in the experimental preclinical setting, a preliminary validation in retrospective testing of biomaterials from biobanks or in sporadic clinical studies is mandatory. Finally, well-designed clinical trials are required to prospectively test the proof of concept and confirm the usefulness of the candidate biomarker in independent cohorts.

Only the strict performance of such clinical development plans will allow to adequately assess test characteristics (sensitivity, specificity, negative and positive predictive values) for a given biomarker and/or a combination of biomarkers as a prerequisite for their implementation in clinical practice. All currently suggested surrogate parameters for clinical PD fluid development have to be validated by comparison to their “hard” clinical outcome in sufficiently large case-control cohort studies. For example, about 350 PD patients would be needed to be followed for up to 2 years in an observational cohort study to detect biomarker differences with an effect size of 0.3 (=30% of their standard deviations), based on previously reported incidence rates of PD-related complications.[1,111] In such a trial, follow-up with repeated sample collection is needed until sufficient pre-defined complications (such as peritonitis, ultrafiltration failure or deterioriation of peritoneal membrane transport characteristics) have occurred. Biomarkers would then be assessed in stored samples and compared betweeen PD patients with complications (=cases) and PD-patients who did not develop complications over the same length of observation (=controls).[112]

These validated biomarkers will then form the accepted basis to define a population of high risk for clinical complications, which might particularly benefit from well-defined alternate therapies in the context of precision medicine. Ultimately, any biomarker research needs to incorporate an analysis of user requirements at the earliest possible time point. Any biomarker-based test will be measured by its ability to provide value for money, not only to improve patient outcomes but also to save costs for healthcare systems and generate revenue for industrial partners. Given the relatively small number of PD patients worldwide compared to other patient cohorts those commercial restraints are particularly relevant.

Table 1

Clinical Question	EMERGING BIOMARKER			CURRENT LEVEL OF EVIDENCE	
Is patient at risk / does intervention reduce risk?	**Consensus Suggested Surrogate Biomarker***	**Concept / Hypothesis**	**reflecting patho-mechanism**	**Observed Associations (with reference)**	**Score***
Membrane Failure?	IL-6, IL-8	Peritoneal levels of IL-6 and IL-8 depend on basal activity of peritoneal immune cells	**Smoldering Inflammation**	Basal levels of cytokines in PD effluents are significantly associated with changes on peritoneal membrane transport characteristics, basal cytokine levels show no consistent differences between patients treated with different glucose-based PD fluids but significantly increased in PD patients treated with icodextrin containing regimens.[12,21-29]	A
Membrane Failure?	CA-125	CA-125 is produced by mesothelial cells and predominantly actively released into the peritoneal cavity	**Membrane Remodeling**	Peritoneal levels of CA-125 significantly decrease during longterm PD, CA-125 levels are lower with glucose based acidic single-chamber PD fluids than with pH neutral multi-chamber PD fluids or with glucose sparing regimens including PD fluids with alternate osmotic agents. [3,5,30]	A
Membrane Failure?	Advanced oxidized protein products AOPP	AOPP reflect posttranslational modification of proteins in the peritoneal cavity reflecting local stress	**Oxidative Stress**	Peritoneal levels of AOPP increase over dwell time and are higher with glucose based acidic single-chamber PD fluid than with pH neutral multi-chamber PD fluid. Levels of AOPP are correlated with peritoneal membrane transport characteristics.[76-78]	B
Peritonitis?	Ex-vivo stimulated cytokine release	Ex-vivo exposure of peritoneal immune cells to TLR ligands results in maximally stimulated cytokine release	**Impaired Host Defense**	*Ex-vivo* stimulation of peritoneal macrophages isolated from PD effluents results in lower cytokine release in patients dialyzed with glucose based acidic single-chamber PD fluid than with pH neutral multi-chamber PD fluid.[55,61-63]	B
***** all published studies report discovery research data and are scored regarding their confirmation level: **A**: significant association in >3 independent studies with >100 PD patients; **B**: significant association in ≥3 independent studies; **C**: significant association in <3 independent studies					

Figure Legends

Figure 1: Prognostic biomarkers help to identify PD patients who are at high risk of complications (such as peritoneal membrane deterioration or peritonitis) and should receive counteracting interventions (such as novel PD fluids). Predictive biomarkers help to identify those PD patients that are most responsive (or unresponsive) to a given intervention.

Figure 2: Biomarker research defined by a “targeted approach” starts hypothesis-driven from selected candidates, reflecting cellular mechanisms of interest, in the experimental setting and is then translated into the clinical context. Based on current evidence, the consortium selected surrogate biomarkers (given in bold) as endpoints to be assessed in a phase II clinical trial of a novel PD additive (EudraCT2013-000400-42/AT). The “open omics approach” starts with bio-material from well-defined clinical cohorts without any prior selection (non-hypothesis-driven). Clinical phenotypes to be assessed with molecular signatures (PAMS) as biomarkers were divided into pro-inflammatory and peritoneal membrane damage associated phenotypes. The pro-inflammatory phenotype was further divided into acute peritonitis and post-peritonitis triggered chronic inflammation. The membrane damage phenotype was further divided into mesothelial-to-mesenchymal transdifferentiation (MMT) and changes in peritoneal membrane function determined by peritoneal equilibration testing. Ideally, the two approaches have to be applied iteratively and their results have to be integrated to foster successful biomarker research. The definition of biomarkers also reflects the currently available technologies in a given research field. Thus, the introduction of omics approaches and advanced statistical models to the field of PD is a quintessential prerequisite to describe and define future biomarkers in the open approach.

Figure 3: Non-hypothesis-driven biomarker research following an open omics approach is particularly attractive using PD effluent (PDE) as source for sample material. The **cellular fraction** suspended in PDE can be analyzed using transcriptomics (focusing on mRNA) and proteomics techniques. For soluble substances in PDE (**dissolved fraction**), transcriptomics might be particularly attractive for micro RNAs (miRNA). Proteomics techniques can be applied but require prior removal of high abundance proteins and/or enrichment of low abundant biomarkers, and metabolomics techniques can be employed to quantify both endogenous metabolites and small molecules specific to PD fluid exposure. Identified molecules from all omics levels can be used for generation of pathogen associated molecular signatures (PAMS) using statistical modeling techniques.

Disclosure

Funding Sources:
The authors were part of the European Training and Research in Peritoneal Dialysis (EuTRiPD) program, a project funded by the European Union within the Marie Curie scheme (287813).

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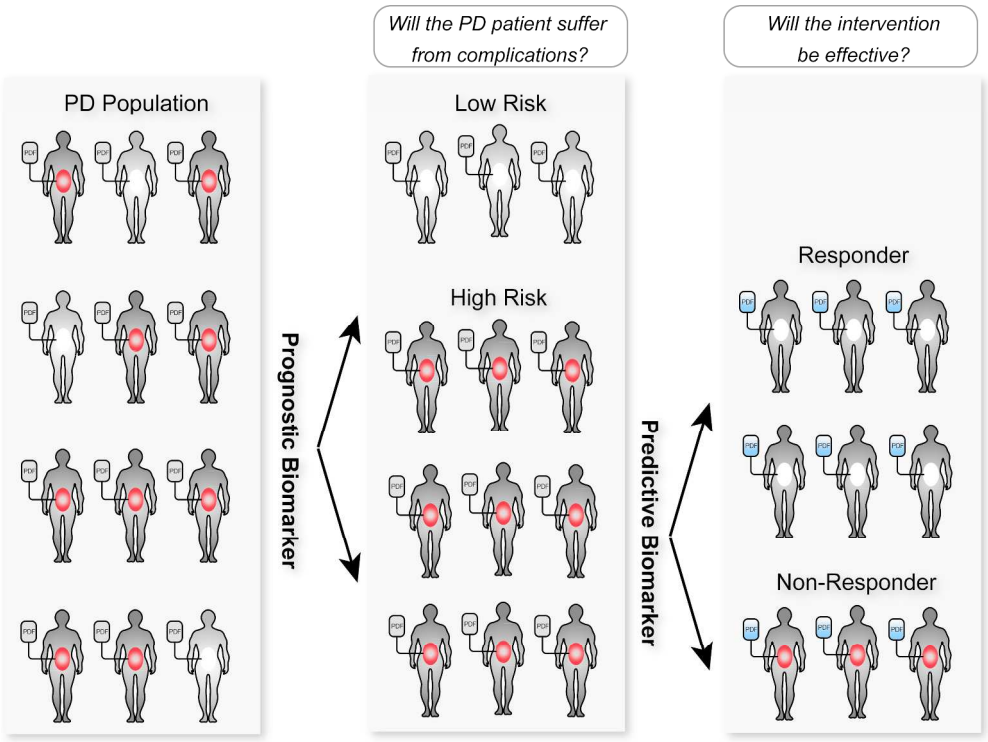

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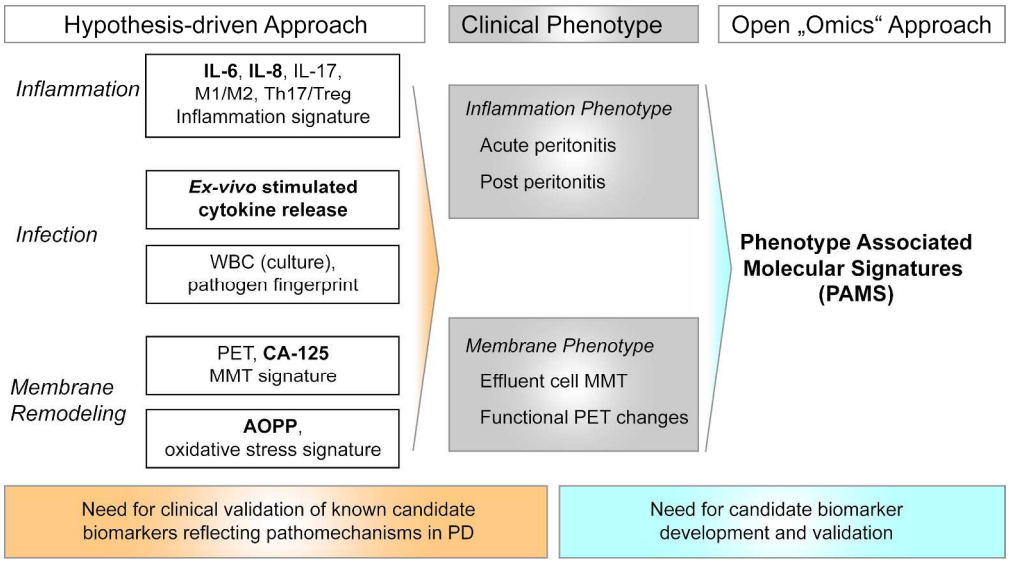

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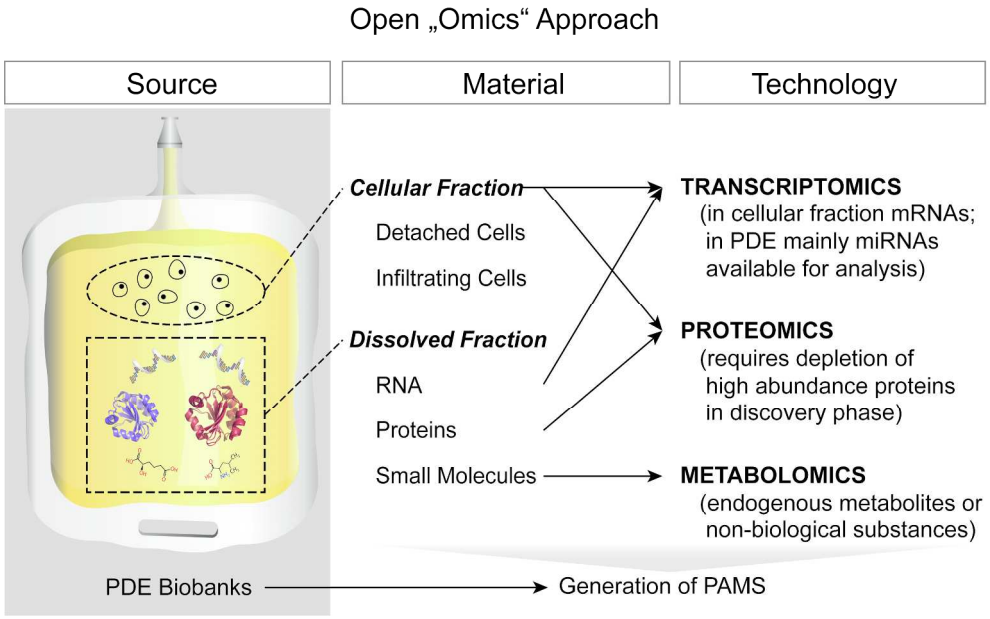

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232x144mm (300 x 300 DPI)