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Reviews

Protein Microarrays for Personalized Medicine

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BACKGROUND: Over the last 10 years, DNA microarrays have achieved a robust analytical performance, enabling their use for analyzing the whole transcriptome or for screening thousands of single-nucleotide polymorphisms in a single experiment. DNA microarrays allow scientists to correlate gene expression signatures with disease progression, to screen for disease-specific mutations, and to treat patients according to their individual genetic profiles; however, the real key is proteins and their manifold functions. It is necessary to achieve a greater understanding of not only protein function and abundance but also their role in the development of diseases. Protein concentrations have been shown to reflect the physiological and pathologic state of an organ, tissue, or cells far more directly than DNA, and proteins can be profiled effectively with protein microarrays, which require only a small amount of sample material.

CONTENT: Protein microarrays have become well-established tools in basic and applied research, and the first products have already entered the in vitro diagnostics market. This review focuses on protein microarray applications for biomarker discovery and validation, disease diagnosis, and use within the area of personalized medicine.

SUMMARY: Protein microarrays have proved to be reliable research tools in screening for a multitude of parameters with only a minimal quantity of sample and have enormous potential in applications for diagnostic and personalized medicine.

The human genome sequencing project provided the basis for the development of DNA microarrays, enabling the massively parallel analysis of mRNA production rates and single-nucleotide polymorphisms. After completion of the Human Genome Project in 2003, DNA microarray technologies developed very quickly and have since evolved into robust and reliable genomics-research tools. The systematic multivariate analysis of genome-wide expression data sets allows evaluation of correlations between gene expression patterns and the state and progression of disease, making possible patient-specific treatment based on a patient's individual genetic profile, an area that is known as "pharmacogenomics" or "personalized medicine" (Fig. 1) *(1, 2)*. Detailed information about a patient's genotype or gene expression profile can be used to stratify disease state, choose appropriate medication, adjust drug dosage according to a patient's requirements, or initiate preventive treatment. Personalized medicine has the ultimate goal of successfully implementing the 5 Rs: "right patient/target, right diagnosis, right treatment, right drug/target, and right dose/time" *(3)*. This goal can be achieved, however, only by combining genomic knowledge with traditional clinical approaches, the patient's personal medical and family history, and relevant clinical data, such as imaging and in vitro diagnostics results.

Gene expression analysis is still not routinely applied in personalized medicine, although gene expression profiling has generated numerous hypotheses relating to tumorigenesis and has provided both potentially prognostic and predictive signatures. Owing to posttranslational modifications such as glycosylation, phosphorylation, and acetylation, proteins are more heterogeneous than the genes encoding them, and this heterogeneity often does not correlate with protein production. Posttranslational modifications extend the function of proteins in terms of recognition, signal transduction, and proteolysis. These processes regulate cell differentiation and/or proliferation. Proteins are also differentially produced in diseases. Many drugs used for treating disease are therefore directed against protein targets, such as protein kinases, cytokines, receptors, or their substrates, that have been shown to play a role in disease development *(4)*. Proteomics analyses therefore provide a more direct way of generating relevant data sets to improve our understanding of diseases at the level of the individual patient. The analysis of protein panels or even entire signaling networks can contribute to increasing the insights into disease development and progression on the molecular level, which in turn can enable the disease diagnosis at the individual level and the adjustment of therapies to the requirements of individual patients. Such detailed analyses require high-throughput multiplexed proteomics techniques capable of screening a multitude of parameters

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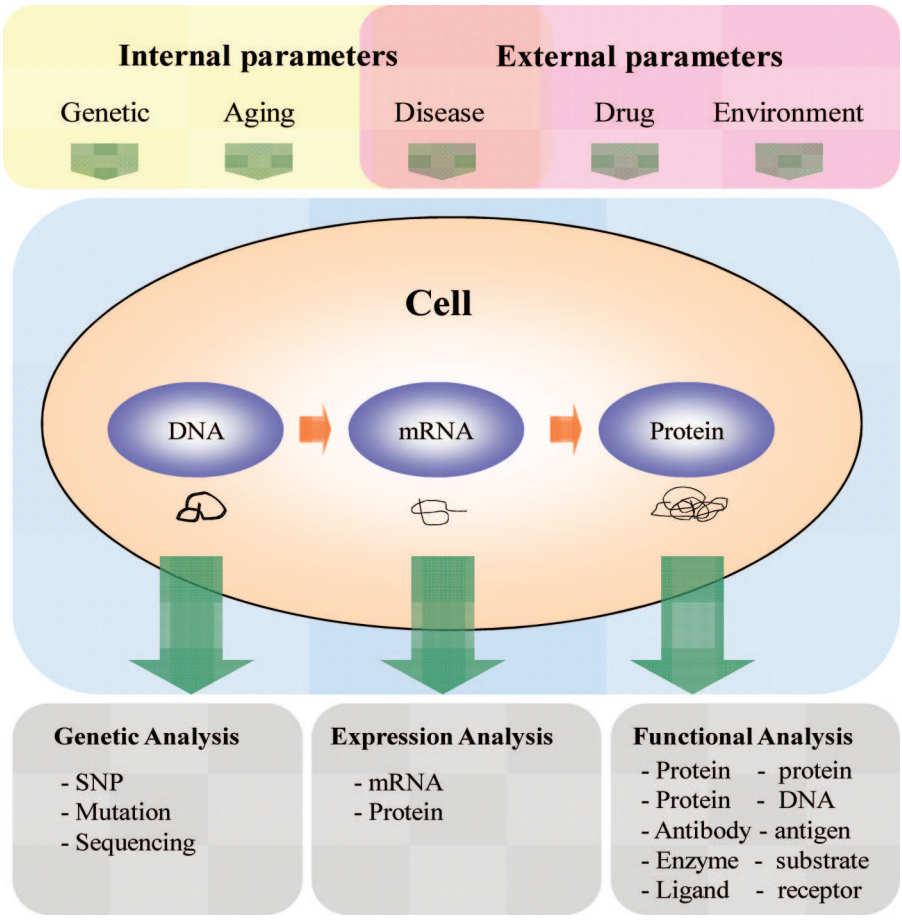

Fig. 1. Influence of internal and external parameters on the physiological state of a cell.

Microarray technology can be applied to monitor intracellular mechanisms of gene expression and protein production. DNA microarrays are used for gene and mRNA analyses, and protein microarrays are used for the analysis of protein production and function.

from minimal amounts of sample materials. Protein microarrays have therefore become a very active research area (Fig. 1) *(5–8)*. Protein microarray–based assays can be grouped according to different formats and types of applications (Figs. 2 and 3).

Forward-phase protein microarray (FPPM)[2] assays are the most frequently used formats. They consist of an array of well-defined immobilized capture molecules that allow the simultaneous analysis of a large number of different parameters in a sample. Examples of FPPM assays are antibody microarrays, which are used to identify and quantify target proteins of interest, and fusion protein arrays consisting of immobilized sets of proteins, which are used to study the interactions between proteins and immobilized binding molecules, such as proteins, peptides, low molecular weight compounds, oligosaccharides, or DNA. FPPM assays involve the immobilization of capture molecules (peptides, proteins, or antibodies) on a solid surface in rows and columns (microarray format) to capture the corresponding analytes present in complex samples (serum, plasma, or cell culture supernatant). The bound analytes are visualized either by direct labeling of the analytes or via a labeled secondary antibody. Standard

[2] Nonstandard abbreviations: FPPM, forward-phase protein microarray; RPPM, reversed-phase protein microarray; CA-125, cancer antigen 125; EGFR, epidermal growth factor receptor; apo A1, apolipoprotein A1; MIP-1α, macrophage inhibitory protein 1α; IL-6, interleukin-6; SSRP1, structure-specific recognition protein 1; SARS, severe acute respiratory syndrome; EGF, epithelial growth factor; HGF, hepatocyte growth factor; RANTES, regulated upon activated, normally T-expressed and presumably secreted; VCAM-1, vascular cell adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; IBD, inflammatory bowel disease; IP-10, 10-kDa interferon γ–inducible protein; FDA, Food and Drug Administration; ANCA, antineutrophil cytoplasmic antibody.

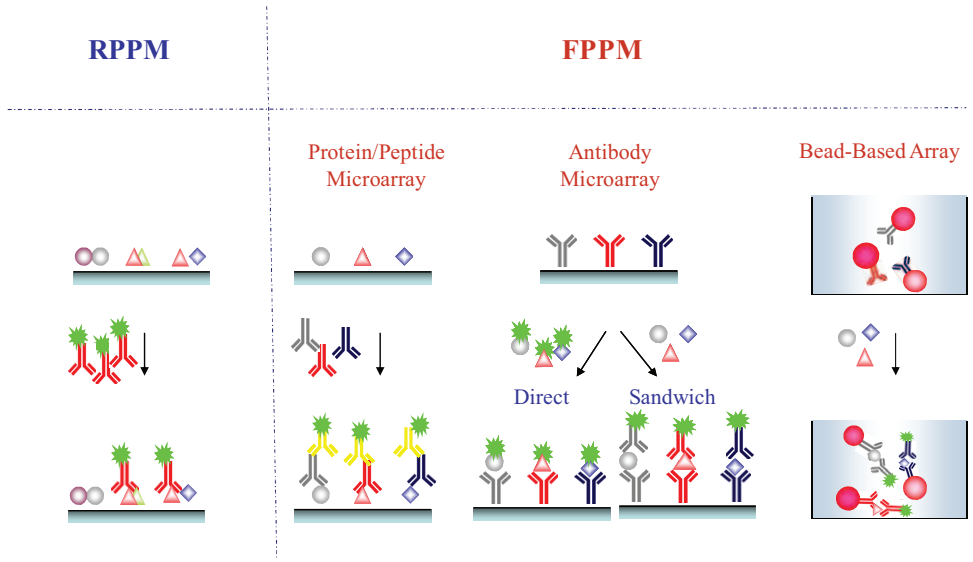

Fig. 2. Protein microarray formats.

RPPMs can be used to measure distinct sets of parameters in a large collection of tissue or cell lysates or to sample fractions immobilized in an array format on a solid support. FPPMs are based on a direct-labeling or a sandwich immunoassay approach and are used for the simultaneous analysis of different parameters from different samples.

detection methods include fluorescence, chemiluminescence, and colorimetry. Planar microarray–based systems are perfectly suited to generate high-density protein microarrays that screen for a large number of analytes in a single experiment. Bead-based array systems provide an interesting alternative to planar microarrays, especially when the number of parameters to be analyzed is comparably low. Such bead-based assay systems are flexible, robust, and more advanced with respect to automation and enable scientists to screen thousands of samples within a short time *(10, 11)*. Many commercial FPPM products are now available, including assays for cytokines and chemokines, cancer biomarkers, and molecules involved in signaling pathways (Tables 1 and 2).

The other assay format, the reversed-phase protein microarray (RPPM), consists of a multitude of different samples, such as tissue or cell lysates, that are immobilized as spots in rows on a solid support. Each microspot contains the entire proteome of a tissue or cell. Highly specific antibodies are used to simultaneously screen these spots for the presence or absence of distinct target proteins. This approach allows the identification of sets of proteins produced in large collections of tissue or cell samples (Figs. 2 and 3) *(11, 12)*. Typically, the molecules of interest, such as transcription factors or signaling molecules, are generally present only in low quantities. The low abundances of such molecules therefore require high-sensitivity detection methods, such as tyramide signal amplification or planar waveguide technology *(13, 14)*. RPPMs have been successfully used in studies of cancer development, both in cells and in patient samples. Table 3 summarizes the main characteristics of the different protein microarray formats.

The remainder of this review covers the current progress in the use of protein microarrays for biomarker discovery and validation, reviews their use in in vitro diagnostics, and discusses the use of protein microarrays in the field of personalized medicine.

Protein Microarrays and Personalized Medicine

Traditional technologies for the analysis of DNA, mRNA, and proteins can analyze only 1 parameter at a time and therefore are limited in throughput when a large number of parameters need to be identified. The implementation of DNA and protein microarrays has led to a tremendous acceleration in biomarker discovery. The ultimate goal is to identify sets of disease-specific biomarkers and then to combine them with a robust screening technology. This approach will allow clinicians to screen patients before prescribing a particular drug in order to exclude potential adverse effects or determine the most suitable dose. Besides making improvements to this new technology, researchers must also identify and validate appropriate sets of biomarkers and subsequently integrate them into

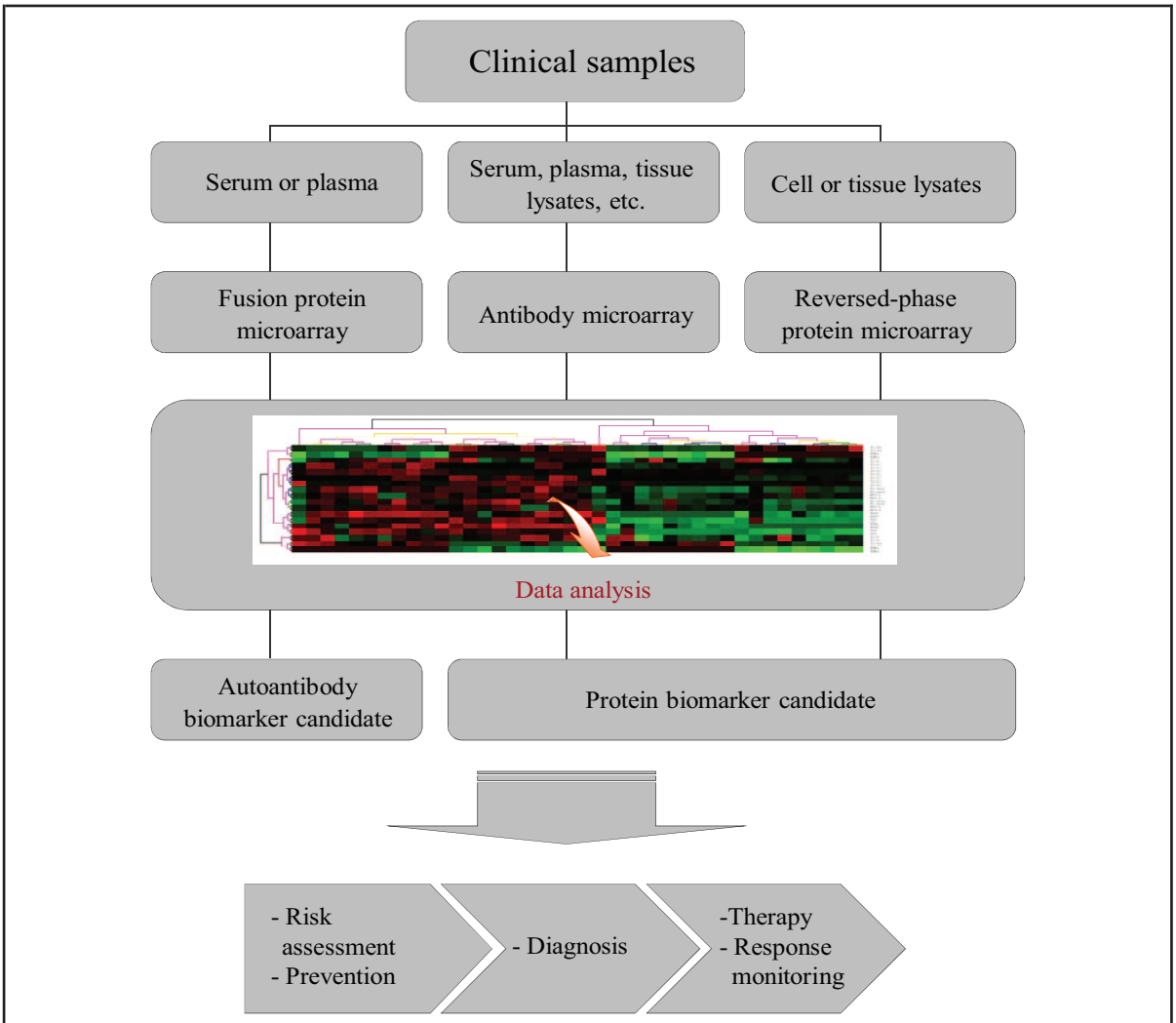

Fig. 3. Work flow of protein microarray applications for biomarker discovery and their application in personalized medicine.

healthcare services. Fig. 3 summarizes the work flow of biomarker discovery with protein microarrays and the anticipated application of protein microarray–based assays for personalized medicine.

Protein microarrays have recently demonstrated their huge potential in the field of biomarker discovery. RPPMs have been used to identify biomarker candidates in cancer patients that show changes in concentration that correlate with the state and progression of disease *(15–20)*. Sandwich immunoassays have proved to be an excellent method for accurately quantifying biomarker candidates. Multiplexing can easily be achieved with bead-based technologies. High-throughput multiplexed immunoassays are perfectly suited for screening cancer biomarkers in a large cohort of clinical samples *(21, 22)* and provide insights into the details of the production of plasma or tissue proteins, thereby furnishing detailed information about a patient's physiological and pathologic condition. The use of sophisticated bioinformatics tools allows the identification of biomarker patterns that can then be used for diagnostic purposes. Data sets generated with multiplexed immunoassays can be integrated into multivariate diagnostic models with such algorithms as K-nearest neighbor and logistic regression. Such analyses can markedly improve the diagnostic sensitivity and specificity of cancer diagnostics compared with what was previously possible with single-parameter analysis *(23–25)*. Recently, Visintin et al. used a 6-plex multiplexed sandwich immunoassay [leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and cancer antigen 125 (CA-125)] to

Table 1. Commercially available miniaturized and parallelized immunoassays based on planar microarray platforms.

Company	Products	Applications
Arrayit	PlasmaScan Antibody Microarrays	Comparative protein profiling
Clontech Laboratories	Ab Microarray 500 & Express Buffer Kit	Comparative protein profiling
EMD Chemicals	InnoCyte 96-well cell adhesion array	Multiplexed immunoassay, cell adhesion
Millipore	EpiTag™ phosphorylation profiling chips	Phosphorylation profiling
Eurogentec	Antibody microarrays	Comparative protein profiling, phosphorylation profiling
Full Moon BioSystems	Antibody microarrays	Comparative protein profiling, phosphorylation profiling
GenTel BioSciences	APiX and PANDEIA antibody microarray multiplexed immunoassays	Comparative protein profiling
Hypromatrix	Signal Transduction AntibodyArray™	Comparative protein profiling, protein–protein interactions, phosphorylation profiling
Invitrogen	ProtoArray® Human Protein Microarray	Autoantibody profiling, protein–protein interactions
Panomics	Human antibody arrays	Multiplexed immunoassay
R&D Systems	MAP Kinase Array Kit, Proteome Profiler Human Pluripotent Stem Cell Array Kit (antibody array)	Comparative protein profiling, protein phosphorylation
Randox Laboratories	Biochip immunoassays	Multiplexed immunoassay
RayBiotech	Phosphorylation antibody arrays, quantitative antibody arrays	Comparative protein profiling, multiplexed immunoassay
Sigma-Aldrich	Antibody Microarray - XPRESS Profiler725 Kit	Comparative protein profiling
Spring Bioscience	Antibody microarrays	Comparative protein profiling
Quansys Biosciences	Multiplexed ELISA assay	Multiplexed immunoassay
Thermo Fisher Scientific	Thermo Scientific Pierce® antibody array	Multiplexed immunoassay
US Biomax	Antibody microarray	Comparative protein profiling

screen plasma from patients with ovarian cancer. Use of this 6-plex panel to screen samples from 362 healthy controls and 156 patients with newly diagnosed ovarian cancer screened achieved a 95.3% diagnostic sensitivity and a 99.4% diagnostic specificity *(23)*. Amonkar et al. identified an 11-plex panel [CA-125, CA 19-9, soluble epidermal growth factor receptor (EGFR), C-reactive protein, myoglobin, apolipoprotein A1 (apo A1), apo CIII, macrophage inhibitory protein 1α (MIP-1α), interleukin-6 (IL-6), IL-18, and tenascin C] and used it to distinguish plasma samples from patients with ovarian cancer from samples from patients with benign conditions. The screening of 176 patients and 187 controls revealed that the 11-plex achieved a diagnostic sensitivity and specificity as high as 90% *(24, 25)*. In contrast, a single parameter, CA-125, currently achieves a diagnostic sensitivity of $<$60% in early stages of the disease *(23)*. These candidate biomarker

Table 2. Commercially available miniaturized and parallelized immunoassays based on bead-based array platforms.

Company	Products	Applications
Bio-Rad Laboratories	Bio-Plex x-Plex assays	Multiplexed immunoassay
EMD Chemicals	WideScreen™ biomarker assays	Multiplexed immunoassay
Invitrogen	Multiple cytokine panel assays	Multiplexed immunoassay
Millipore	MILLIPLEX™ MAP cytokine assays	Multiplexed immunoassay
Panomics	Procarta cytokine-profiling assays	Multiplexed immunoassay
R&D Systems	Human Fluorokine® MAP assays	Multiplexed immunoassay
Rules-Based Medicine	HumanMAP® multiplexed assays	Multiplexed immunoassay

Table 3. Characteristics of the different protein microarrays.

		FPPM	
	RPPM	Planar microarray	Bead-based array
Immobilization	Cell or tissue lysates	Peptide, protein, or antibody	Peptide, protein, or antibody
Labeling of analyte	Without	With or without	With or without
Quantification	Relative	Relative or absolute	Relative or absolute
Number of spots	>1000	>1000	≤100
Throughput	Low	Low	High
Automation	Low	Low	High

panels require further validation with larger patient cohorts, however, before they can reliably be used in routine clinical diagnostics. The increasing list of biomarker candidates shows that protein microarrays have a huge potential to contribute to the development of individualized medical therapies. The knowledge generated with such protein microarrays not only will help clinicians adapt therapies to individual patient requirements but also will help minimize the risk of adverse effects caused by an incorrectly chosen drug or drug dose.

Protein Microarrays for Biomarker Discovery

Protein microarrays enable massively parallel quantification of biomarker candidates from large numbers of clinical samples in a very short time. The hierarchical clustering analysis of these data sets can reveal signatures that correlate with the state of the disease *(26)*. The 3 major applications of protein microarrays for biomarker discovery include the data-driven approach, the knowledge-driven approach, and the systems biology–driven approach.

THE DATA-DRIVEN APPROACH

The data-driven approach involves a proteome-wide analysis to identify a correlation between protein concentrations and a particular disease state. This approach is unbiased in that no assumptions are made about the proteins that might be involved in the disease process; however, the protein microarray approaches developed for global analysis of protein concentrations are still in their infancy. Combining the current antibody arrays with a direct sample-labeling strategy allows the analysis of only a limited number of parameters. Despite all the efforts made in recombinant antibody technology and classic antibody generation, high-quality antibodies directed against every single protein of interest are still not available *(27, 28)*.

Nevertheless, high-throughput techniques for protein analysis have been used to generate whole-proteome arrays consisting of thousands of recombinant proteins *(29–32)*. High-density protein microarrays are a data-driven approach that enables the identification of new autoantibodies that are specific for a variety of diseases *(7, 33)*. Hudson et al. used a protein microarray containing 5005 human proteins to identify autoantibody sets capable of recognizing 94 antigens in the sera of patients with ovarian cancer *(30)*. Four of these antigens were further confirmed with immunoblot and tissue microarray analyses. The researchers found that lamin A/C, structure-specific recognition protein 1 (SSRP1), and Ral-binding protein 1 were produced in larger quantities in cancer tissue than in control tissues. Furthermore, the diagnostic specificity and sensitivity of the combined use of lamin A/C and SSRP1 performed better than tumor marker CA-125 alone in identifying ovarian cancer.

The same approach was used to assess the immune response to pathogenic proteins. Zhu et al. generated a coronavirus protein microarray containing 82 purified proteins from severe acute respiratory syndrome (SARS) coronavirus and 5 additional coronaviruses, with the aim of monitoring the immune response of patients infected with SARS. Approximately 400 serum samples from the Canadian SARS outbreak were screened. These samples included those from confirmed SARS coronavirus cases, patients with respiratory illnesses, and asymptomatic healthcare workers. Bioinformatics data analysis allowed classification of the sera with 91% accuracy (90% diagnostic sensitivity and 93% diagnostic specificity) *(34)*. This study demonstrated very well that protein microarrays can be used for large-scale identification of virus-specific antibodies in sera. Schmid et al. generated a protein microarray consisting of 251 proteins (92% of the vaccinia virus proteome). The screening of the sera obtained from vaccinated individuals (n = 20) and nonvaccinated individuals (n = 20) revealed that the primary antibody response to individual vaccinia proteins varied from individual to individual, whereas the total number of proteins recognized by antibodies was

only slightly altered after the second vaccination *(35)*. These data sets proved that protein microarrays could measure humoral immune responses to vaccines and provide a straightforward approach to evaluating newly developed vaccines.

THE KNOWLEDGE-DRIVEN APPROACH

In a knowledge-driven approach, which is a targeted proteomic approach, biomarker candidates relevant to a certain disease are defined according to existing scientific knowledge. The drawbacks of such an approach, however, are that the outcome is only as good as the state of scientific knowledge and that there is a risk, of course, of missing unknown biomarkers. Nevertheless, the knowledge-driven approach is still the major strategy when screening for such analytes as acute-phase proteins, signaling molecules, and autoantibodies *(36–38)*. Cytokines and chemokines, which regulate a variety of cell activities, including cell migration, proliferation, and apoptosis, and therefore play an important role in inflammatory diseases and different cancers *(39–41)*, are the major proteins used in such studies *(42, 43)*.

Diagnostic biomarkers. Nodular thyroid disease, which affects 50% of individuals who are 50 years old, is characterized by the presence of single or multiple nodules within the thyroid gland. More than 90% of thyroid nodules are not harmful or cancerous, but if thyroid cancer is diagnosed, treatment should begin as soon as possible. Fine-needle biopsy is the standard diagnostic method, but 30% of results generated with the biopsy approach are not satisfactory. Linkov et al. used the bead-based xMAP technology to identify plasma patterns of 19 cytokines, chemokines, and growth factors that correlate with benign and malignant thyroid conditions *(44)*. Univariate analysis revealed that 5 factors [epithelial growth factor (EGF), hepatocyte growth factor (HGF), IL-5, IL-8, and RANTES (regulated upon activation, normally T-expressed and presumably secreted)] enabled individuals with thyroid disease to be distinguished from the control group. Further multivariate analysis revealed a panel of 4 parameters (IL-8, HGF, monocyte-induced γ interferon, IL-12p40) that can discriminate between benign and malignant disease states (area under the ROC curve, 0.81; 95% CI, 0.65–0.90). This result demonstrates that protein arrays that define panels of plasma/serum biomarkers are promising tools to support the diagnosis of nodular thyroid disease.

Sauer et al. used protein arrays to profile fine-needle biopsies from breast cancer patients to identify a panel of biomarkers for specific subgroups of breast cancer patients *(45)*. Lysates prepared from large-core needle biopsies of invasive breast carcinomas were analyzed with bead-based immunoassays with the overall aim of evaluating the production of 54 preselected proteins. The results revealed that the profiles of 5 proteins in the tumor (fibroblast growth factor 2, Fas, Fas ligand, tissue inhibitor of metalloproteinase 1, and RANTES) differed considerably between the patient groups with and without axillary lymph node metastasis. Kim et al. used a similar approach to distinguish breast cancer patients from healthy volunteers *(46)*. Thirty-five analytes were selected from 4500 previously screened plasma samples obtained from patients with various types of cancer. Three biomarkers (EGF, soluble CD40 ligand, pro–apo A1) were identified at increased concentrations in plasma samples from the breast cancer group. Six biomarkers that showed a decreased plasma concentration [high molecular weight kininogen, apo A1, soluble vascular cell adhesion molecule 1 (VCAM-1), plasminogen activator inhibitor 1, vitamin D–binding protein, and vitronectin] were also identified. Different algorithms were then used to generate diagnostic models, which allowed breast cancer patients to be discriminated from the healthy controls with a high level of diagnostic accuracy (91.8% with random forest analysis, 91.5% with support vector machine analysis, and 87.6% with linear discriminant analysis).

Prognostic biomarkers. There is a huge demand for the identification of sepsis-specific biomarkers that can reflect the state and severity of this inflammatory disease. Bozza et al. used miniaturized sandwich immunoassays to evaluate the predictive values of cytokine production patterns in a sepsis time course *(47)*. They found that some cytokine concentrations increased as the severity of inflammation and organ dysfunction increased. With regard to the severity of organ dysfunction, the concentrations of 2 the 17 evaluated cytokines, IL-8 and monocyte chemoattractant protein 1 (MCP-1), measured on day 1 correlated significantly with the Sequential Organ Failure Assessment score. Increased plasma concentrations of IL-6, IL-8, and granulocyte colony-stimulating factor within the first 24 h correlated with organ dysfunction, which failed to recover on day 3. The concentrations of 6 cytokines (IL-1β, IL-4, IL-6, IL-8, MCP-1, and granulocyte colony-stimulating factor) allowed prediction of early mortality.

To identify serum biomarkers of early and late inflammatory bowel disease (IBD), Torrence et al. used a mouse model (*mdr1a*$^{-/-}$ mice) for IBD triggered by infection with *Helicobacter bilis (48)*. The multianalyte profiling data revealed an increase in 5 plasma proteins [IL-11, IL-17, 10-kDa interferon γ–inducible protein (IP-10), lymphotactin, MCP-1, and VCAM-1] in early-stage IBD. Eleven proteins (IL-11, IP-10, hapto-

globin, matrix metalloproteinase 9, MIP-1γ, fibrinogen, IgA, MIP-3β, VCAM-1, apo A1, and IL-18) displayed higher concentrations in a late stage of the disease. The concentrations of all these biomarkers except apo A1 correlated with the histopathologic scores. Appropriate antibiotic treatment improved the clinical signs of IBD and led to decreases in the mean plasma concentrations of many of the biomarkers. Such IBD animal models seem to be suitable for identifying noninvasive biomarkers for monitoring IBD progression, while at the same time increasing our understanding of IBD pathogenesis, which is a major prerequisite for the development of new therapies. The observations from animal models need to be confirmed with human IBD plasma samples, however, before definite statements can be made on the suitability of such biomarkers.

Predictive biomarkers. Twenty to forty percent of all rheumatoid arthritis patients do not respond to treatment with tumor necrosis factor α–blocking agents such as etanercept. These inappropriate treatments not only are costly but also have severe side effects in these patients *(49, 50)*. Therefore, the identification of biomarkers that can predict a clinical response to an anti–tumor necrosis factor α treatment would be extremely useful. Panels of cytokine production in plasma may enable the discrimination of responders from nonresponders. Fabre et al. profiled plasma cytokines in rheumatoid arthritis patients undergoing etanercept therapy *(49)*. The Evidence Biochip Array (Randox Laboratories) was used to measure 10 proinflammatory and 2 antiinflammatory cytokines in serum samples collected on days 0 and 90. The researchers found that high plasma concentrations of MCP-1 and EGF were associated with a response to etanercept. In addition, the combination of C-reactive protein and EGF proved to be a good way of predicting the effect of etanercept treatment at 3 months (diagnostic sensitivity, 87.5%; diagnostic specificity, 75%).

Allen et al. observed that the plasma concentrations of nuclear factor κB–modulated cytokines and growth factors (IL-6, IL-8, growth-regulated oncogene 1, vascular endothelial growth factor, and HGF) changed in patients with head and neck squamous cell carcinoma who had been treated with chemotherapy and radiotherapy. Plasma parameters were quantitatively analyzed in samples from patients with a diagnosis of stage III/IV oropharyngeal carcinoma to discover whether the plasma parameters of these proteins correlated with treatment response, relapse, or survival *(51)*. The patients were treated with combined chemotherapy and radiotherapy, and the relationship between cytokine plasma concentrations and the survival rate was investigated with Cox proportional hazards models and Kaplan-Meier survival analysis. The detailed analysis of the data revealed that the concentrations of these cytokines correlated with both the response to therapy and poorer cause-specific survival. A history of nonsmoking and higher vascular endothelial growth factor concentrations also correlated with an increased survival rate. This approach is envisioned to be extendable to other cancers with a poor predicted outcome, because the monitoring of longitudinal changes in cytokine concentrations can complement the information provided by clinical monitoring and imaging approaches.

Signaling pathway profiling. Kinases are a very interesting group of molecular targets for cancer treatment because they are involved, among other things, in the regulation of cell proliferation and differentiation. A systematic analysis of such signaling proteins in samples of individual tumors may reveal multiple causative players during cancer progression *(14, 52, 53)*. Laser capture microdissection can be used to resolve the problem of tissue heterogeneity by enabling the isolation of pure tumor cells from tissue sections *(14)*. Wulfkuhle et al. used RPPMs in combination with laser capture microdissection technology to carry out a systematic analysis of cellular signaling in breast cancer tissue and metastatic lesions *(20)*. Twenty-five surgical samples of human breast cancers were screened for the presence of 90 signaling molecules. It was possible to define subgroups of samples of primary breast tumors and metastatic lesions by their protein production or degree of phosphorylation on the basis of the pathway-specific signaling profiles, such as EGFR family signaling, AKT/mTOR pathway activation, c-kit/abl growth factor signaling, or ERK pathway activation. The authors proposed that the detailed characterization of signaling activity has a huge potential with respect to the design of patient-tailored therapeutic regimens. With a similar approach, Sheehan et al. profiled the signaling activities of epithelial and stromal compartments of colon carcinomas. The authors inferred from the production patterns of >60 signaling molecules that a coordinated cross talk exists between epithelium and stroma in cancer, suggesting an epithelial–mesenchymal transition. Such cross talk could produce a therapy-resistant tumor epithelium. Such protein microarray–generated findings could have therapeutic implications for the treatment of colon cancer *(54)*.

THE SYSTEMS BIOLOGY–DRIVEN APPROACH

Systems biology is an interdisciplinary research area that focuses on the systematic study of complex interactions in biological systems and the development of mechanistic models for predicting the behavior of the

dynamic system. Compared with reductionist approaches, which study smaller spatial scales or organizational units to understand the nature of complex systems, the systems biology–driven approach is based on the integration of large genomic and proteomic data sets in combination with a broad biological knowledge and computational techniques. The large number of parameters, variables, and constraints in cellular networks requires the use of numerical and computational techniques. The efforts made in systems biology approaches are aimed at gaining detailed insights into all factors in a living cell and the changes that occur during disease progression *(55)*. Protein microarrays have proved to be one of the large-scale measurement methods capable of delivering experimental data sets in the quantities required for computational approaches *(56)*. Knickerbocker et al. analyzed early mortality in patients undergoing kidney dialysis. The researchers applied protein microarrays to quantify sets of plasma parameters in patient samples and combined these data sets with such clinical variables as body mass index, diastolic blood pressure, underlying disease, and method of vascular access *(57)*. The authors were able to predict early mortality in patients undergoing kidney dialysis when the molecular markers were combined with the clinical variables. A systems biology–based analysis of this kind enables an individualized prognosis and supports clinicians in the choice of treatment regimens best suited for an individual patient.

Protein Microarrays for Biomarker Validation

Within the last decade, proteomics technologies have led to the identification of a huge number of biomarker candidates; however, the current limitation is the validation of biomarker candidates, only a few of which have been successfully validated. These markers include α-fetoprotein, carcinoembryonic antigen, and prostate-specific antigen *(58, 59)*. Numerous aspects of the validation process need to be improved. The current high-output technologies used for the discovery of biomarkers are low-throughput methods *(60, 61)*. Therefore, novel technologies with a higher throughput and a higher analytical sensitivity are required; such technologies must also take into account the fact that only small quantities of sample are typically available, particularly in the case of clinical samples *(61)*. Protein microarrays have proved to be an excellent approach for biomarker-validation studies. Over the last few years, different automation concepts have been developed for protein arrays. One of the most advanced formats is the bead-based systems, for which different companies have developed automated systems *(62)*. In addition, planar protein microarrays have been adapted to the 96-well microtiter plate format, which allows microarrays to be processed with standard pipetting robots or multichannel pipettes *(22, 57, 63)*. Several diagnostics companies have developed other protein technology platforms, such as the Randox Evidence biochip system and the IMPACT professional diagnostics platform (Roche Diagnostics) *(64)*.

The major challenge in biomarker validation is the availability of high-quality capture molecules, e.g., antibodies *(22)*. Despite tremendous efforts in the development of new methods to produce high-quality capture molecules such as aptamers or recombinant antibodies *(36, 65, 66)*, their potential value in biomarker validation still needs to be demonstrated.

Protein Microarrays and In Vitro Diagnostics

Protein microarrays are excellently suited for diagnostic purposes *(12, 67)*. Several diagnostic protein microarray products have been cleared by the US Food and Drug Administration (FDA) or CE-marked for use in the EU *(9)*. The diagnosis of autoimmune diseases is the focus of most of these protein microarrays, including the AtheNA Multi-Lyte® Test System (Zeus Scientific; exclusively marketed by Inverness Medical Professional Diagnostics), the Bio-Plex™ 2200 system (Bio-Rad Laboratories), the Immuno Solid-phase Allergen Chip (VBC Genomics Bioscience Research), and the QUANTA Plex™ ANCA Profile (INOVA Diagnostics,). The AtheNA Multi-Lyte ANCA Test System, which performs multiple assays simultaneously from a single sample in a single well, can perform qualitative or semiquantitative detection of IgG-class antibodies to 2 separate antineutrophil cytoplasmic antibody (ANCA) antigens (myeloperoxidase and oroteinase 3). The intent is to aid the diagnosis of a number of autoimmune vasculitic disorders characterized by increased concentrations of ANCAs. The company's AtheNA Multi-Lyte® ANA Test System simultaneously performs antinuclear antibody screening and reflex testing for 9 specific autoantibodies (SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, centromere B, and histone) in a single well.

Focus Diagnostics developed 2 serologic tests (Plexus™ HerpeSelect® 1 and 2 IgG test kit; Plexus EBV Multi-Analyte Diagnostics serology test kit) for the diagnosis of infectious diseases. The FDA cleared these tests in 2007 and 2008, respectively. The Plexus HerpeSelect 1 and 2 IgG test kit is the first multiplex, type-specific herpes simplex virus serology test that uses Luminex xMAP technology. It detects antibodies to herpes simplex virus types 1 and 2 and assists clinicians in choosing appropriate treatment and counseling. The second test, the Plexus EBV Multi-Analyte Diagnostics serology test kit, is used for detecting the presence or absence of IgG and IgM antibodies in human blood

serum. It is used for the diagnosis of Epstein–Barr virus infection and Epstein–Barr virus–associated infectious mononucleosis. The Randox Evidence Biochip Array system can perform miniaturized multiplexed immunoassays in a macroarray format containing 25 features, with chemiluminescence used as readout. This fully automated clinical biochip analyzer screens patient samples for a variety of markers specific to fertility, cardiac disease, tumors, cytokines, growth factors, cell adhesion molecules, thyroid function, or drug abuse. The company's drug abuse panel has received FDA clearance; the other assays are currently being evaluated by the FDA *(68)*.

It can be envisaged that a growing number of biomarkers will be defined and applied to diagnostic tests in the future. This goal requires that appropriate quality controls be put in place and that the release of nonrequested test data and the handling of the diagnostic results be implemented and regulated *(69, 70)*. These biomarker patterns will provide more individualized information, which will then provide support to clinicians in the diagnosis and selection of optimal therapies.

Perspectives

Multiplex protein assays will become more widely accepted and implemented in different diagnostic areas. Further advances in protein microarray technology will enable clinicians to use just 1 drop of blood to screen patients for relevant pathologic information in the doctor's office before a particular drug is prescribed. The FDA's Critical Path Initiative and the Innovative Medicines Initiative, a partnership between the European Community and the European Federation of Pharmaceutical Industries and Associations, are strategies for supporting the development of biomarker assays, both for traditional diagnostic purposes and to support the drug-development process *(71, 72)*.

These publicly supported efforts have been set up to back the collaboration between companies, policy makers, research institutes, and hospitals. Such activities have the potential to accelerate the development of protein microarrays for diagnostic purposes and will lead to personalized medicine becoming a reality. Nevertheless, proof will be required that a medical need exists for diagnostic protein arrays, that they provide therapeutically relevant results, and that they generate an overall cost reduction before such arrays can finally fulfill the high expectations they have raised.

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