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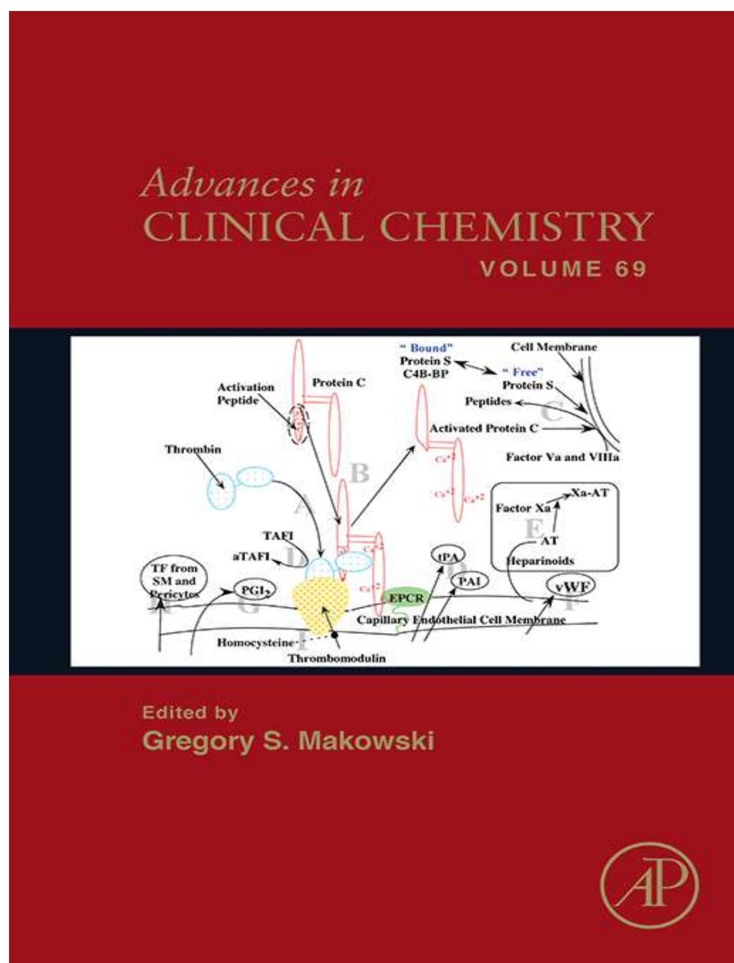


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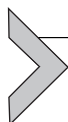

CHAPTER SEVEN

# Antibody Arrays in Biomarker Discovery

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## Abstract

All of life is regulated by complex and organized chemical reactions that help dictate when to grow, to move, to reproduce, and to die. When these processes go awry, or are interrupted by pathological agents, diseases such as cancer, autoimmunity, or infections can result. Cytokines, chemokines, growth factors, adipokines, and other chemical moieties make up a vast subset of these chemical reactions that are altered in disease states, and monitoring changes in these molecules could provide for the identification of disease biomarkers. From the first identification of carcinoembryonic antigen, to the discovery of prostate-specific antigen, to numerous others described within, biomarkers of disease are detectable in a plethora of sample types. The growing number of biomarkers for infection, autoimmunity, and cancer allow for increasingly early detection, to identification of novel drug targets, to prognostic indicators of disease outcome. However, more and more studies are finding that a single cytokine or growth factor is insufficient as a true disease biomarker and that a more global perspective is needed to understand true disease biology. Such a broad view requires a multiplexed platform for chemical detection, and antibody arrays meet and exceed this need by performing this detection in a high-throughput fashion. Herein, we will discuss how antibody arrays have evolved, and how they have helped direct new drug target design, helped identify therapeutic disease markers, and helped in earlier disease detection. From asthma to renal disease, and neurological dysfunction to immunologic disorders, antibody arrays afford a bright future for new biomarkers discovery.

## ABBREVIATIONS

**AD** Alzheimer's disease
**AIDS** acquired immunodeficiency syndrome
**APP** amyloid precursor protein
**CA-125** carbohydrate/cancer antigen 125
**CCL** CC chemokine ligand
**CD** Crohn's disease
**CRC** colorectal cancer
**CSF** cerebrospinal fluid
**CXCL** CXC chemokine ligand
**ECP** eosinophil cationic protein
**EGFR** epithelial growth factor receptor
**ELISA** enzyme-linked immunosorbent assay

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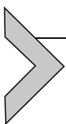
**eNet** elastic net regression algorithm
**$FEV_1$** forced expiratory volume in 1 s
**FGF** fibroblast growth factor
**GI** gastrointestinal
**GRO** growth-regulated oncogene
**GVHD** graft versus host disease
**HC gp-39** human cartilage glycoprotein-39
**HGF** hepatocyte growth factor
**IBD** inflammatory bowel disease
**IFN** interferon
**IL** interleukin
**IL-2Rα** interleukin 2 receptor alpha
**IP-10** interferon gamma-induced protein 10
**mAb** monoclonal antibody
**MCF-7** Michigan Cancer Foundation 7 (breast cancer cell line)
**MCI** mild cognitive impairment
**MCP-1** monocyte chemotactic protein 1
**M-CSF** macrophage colony stimulating factor
**MS** mass spectrometry
**NDC** nondemented controls
**NT-3** neurotrophin-3
**OD** other types of dementia
**OSM** oncostatin M
**PAM** prediction analysis of microarray
**PARC** pulmonary and activation-regulated chemokine
**PLGF** placental growth factor
**PSA** prostate-specific antigen
**QCM** quartz crystal microbalance
**RA** rheumatoid arthritis
**RANTES** regulated upon activation normal T-cell expressed and secreted
**SAM** Significance Analysis of Microarray
**SCGFβ** stem cell growth factor beta
**SLE** systemic lupus erythematosus
**SPR** surface plasmon resonance
**SS** systemic sclerosis
**T1D** type 1 diabetes
**TARC** thymus and activation-regulated chemokine
**TGF-β1** transforming growth factor beta-1
**TNF** tumor necrosis factor
**TNFRI** tumor necrosis factor receptor 1
**VEGF** vascular endothelial growth factor
**VEGFR** vascular endothelial growth factor receptor



## 1. THE ORIGIN OF BIOMARKERS

The ability to identify and classify a physiological phenotype, disease, or biological disorder with a high degree of certainty has been at the

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forefront of medicine since its dawn. Doctors, clinicians, and researchers have long strived to identify better and more accurate methods of identifying medical anomalies. The reasons for this are obvious; to provide a more accurate assessment of an individual's condition via a comprehensive medical diagnosis which will allow for a more accurately administered treatment regimen, and ultimately a more positive prognosis. In many instances, the early, rapid, and accurate assessment of a patient's condition drastically improves long-term outcome and patient survival.

Attempts at medical diagnostics have been documented to have occurred as early as the late 2600s B.C. During this time, the Egyptian architect, engineer, and physician Imhotep wrote what is now called the *Edwin Smith Papyrus*, a medical textbook compiling various information gathered at the time on human physiology, anatomy, diseases, disorders, and corresponding treatments. This text is considered to be the first of its kind to be utilized by physicians for diagnosing human maladies (Fig. 1). One thousand years later the chief scholar, Esagil-kin-apli, of ancient Babylonia wrote the Diagnostics Handbook which emphasized the application of logic, reason, and empirical values when diagnosing a medical disorder. This was followed by an outline and description of the four diagnostic methods of traditional Chinese medicine in the *Yellow Emperor's Inner Canon*. These include inspection, listening/smelling, inquiry (asking questions), and palpation. These methods are still followed today. Yet it was not until the early 1900s that considerable emphasis was placed on first identifying a medical

**Figure 1** The Edwin Smith Papyrus. *Photograph courtesy of Wikimedia Commons.*

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disorder and then attempting its eradication. This was decades before the discovery of DNA as the hereditary key for both health and sickness.

Yet even in the context of the central dogma of DNA, it is perhaps the final protein product which is indeed most telling in relation to the "biomarking" of a medical phenomenon. As proteins are often comprehensively affected during disease progression, it is only at this final posttranslational level of gene expression where the definitive effect of a medical disorder may be best characterized. Therefore, proteomics-based approaches hold much promise with respect to not only the identification of disease-specific biomarkers, but their widespread use in basic research, drug discovery, and clinical diagnostics. The following section outlines examples of both nucleic acid- and protein-based biomarkers as well as other markers and tools which act as valuable determinants of various biological states or medical disorders.

## 1.1 Types of biomarkers

The early underpinnings of medical diagnostics all held one common theme: the presence of symptoms or "markers" which might yield insight into the medical disorder, or even foretell the patient's recovery or demise. "Biological markers," now simply denoted as "biomarkers," are therefore defined as measurable indicators which may define a particular biological state such as a disease, infection, or environmental exposure. There are many types of biomarkers, some of which are now accepted by the medical community as standard and some of which (microRNA—mRNA), are rapidly emerging as powerful markers for biology and disease [1,2]. These biomarkers may take the form of entire cells, molecules, enzymatic activities, antibodies or even minute metabolites such as amino acids. Table 1 briefly outlines the largest impacting preclinical research and clinical diagnostics biomarkers types and any corresponding disease states they are being used for.

## 1.2 Significance of biomarkers in research and clinical diagnostics

The significance of biomarkers goes well beyond patient diagnostics. These hallmarks of human disease may be utilized in the drug discovery process and in preclinical research. The assignment of standards regarding biomarker identity, presence, and activity for the identification or medical disorders or states of physiology provides an invaluable opportunity to optimize and streamline sample analysis. Thus, biomarkers may be used to measure

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**Table 1** Examples of currently studied and utilized biomarkers

| Type of biomarker | Example | Indication | Refs. |
|---|---|---|---|
| Cells | Cancer stem cells<br>Circulating tumor cells (CTC)<br>Lymphocytes | Multiple types of cancer | Woodward, W.A., Sulman, E.P. *Cancer Metastasis Rev.* 2008; 27(3):459–470 [183] |
| Genes (DNA) | Mutations in BRCA1 and BRCA2 | Breast cancer | Narod, S.A., Foulkes, W.D. *Nat. Rev. Cancer* 2004; 4(9):665–676 [184] |
| Gene modification—methylation | Hypermethylation of p16, SOCS1, GSTP1, and CDH1 | Hepatocellular carcinoma | Mah, W.C., Lee, C.G. *Biomark. Res.* 2014; 2(1):5 [185] |
| Gene products—mRNA transcripts (RNA) | CD11c | Prediction of response to anti-TNF monotherapy | Stuhlmuller, B., *et al. Clin. Pharmacol. Ther.* 2010; 87:311–321 [186] |
| Gene products—proteins | Prostate-specific antigen (PSA) | Prostate cancer | Catalona, W.J., *et al. J. Urol.* 1994; 151 (5):1283–1290 [187] |
| Enzymes | Creatine kinase | Myocardial infarction | Gulbis, B., *et al. Clin. Chem.* 1990; 36(10):1784–1788 [188] |
| Peptides | Natriuretic peptide (NT-proANP) | Hypertension | Uusimaa, P., *et al. Acta Cardiol.* 2011; 66(1):21–27 [189] |
| Autoantibodies | Rheumatoid factor | Hepatitis C viral infection | Yang, D.H., *et al. World J. Gastroenterol.* 2014; 20(11):2962–2970 [190] |
| Steroids | Cholesterol | Coronary artery and vascular disease | Rudolf, J., Lewandrowski, K.B. *Clin. Lab. Med.* 2014; 34(1):113–127 [191] |
| Hormones | Cortisol | Depression | Owens, M., *et al. Proc. Natl. Acad. Sci. U. S. A.* 2014; 111(9): 3638–3643 [192] |

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**Table 1** Examples of currently studied and utilized biomarkers—cont'd

| Type of biomarker | Example | Indication | Refs. |
|---|---|---|---|
| MicroRNA | miR-142-3p and miR-181a | Systemic lupus erythematosus (SLE) | Carlsen, A.L., *et al. Arthritis Rheum*. 2013; 65(5):1324–1334 [193] |
| Glycans | *O*-glycan structures on MUC1 | Breast, prostate, and gastric cancers | Albrecht, H., *et al. Cancer Biother. Radiopharm*. 2011; 26(3):261–271. [194] |
| Exosomes | Prostate derived | Prostate cancer | Drake, R.R., *et al. Expert Rev. Proteomics* 2014; 11(2):167–177 [195] |
| Metabolomics | Carbohydrate, lipid, and amino acid metabolisms | Polycystic ovary syndrome | Zhao, Y., *et al. BMC Med*. 2012; 10:153 [196] |

and evaluate normal biological processes, pathogenic disease states, drug toxicity, and drug efficacy to name a few.

### *1.2.1 Normal biological processes*

A great deal of research in basic biology often requires precise and accurate data points with respect to measuring response to a particular stimulus. Biomarkers may act as valuable endpoints for amassing this information in an organized and manageable dataset. At the organismal level, knockout mice are an example of a discrete and specific genetic inactivation, which may have adverse effects on inherent biological processes such as inflammation. An example is a publication on the characterization of presenilin 1 (PS1) and 2 (PS2), highly related transmembrane-domain proteins reportedly involved in the proteolytic processing of amyloid-β precursor protein (AβPP), a protein that if abnormally folded can drive the plaque formation seen in Alzheimer's disease (AD). Through genetic KO of both PS1 and PS2 loci in mice, Jiang *et al.* utilized a mouse inflammation array to measure the expression of 40 cytokines in these mouse's brains [3]. The results revealed that KC and tumor necrosis factor (TNF)-α, among others, were significantly elevated in the brains of dKO versus wild-type mice (Table 2 and [3]). These findings demonstrate the utility of multiplexed antibody array technology for the efficient characterization of biomarker levels in biological specimens as applied to basic biomedical research. Antibody arrays for both

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**Table 2** Modulations in inflammatory mediators in presenilin 1 and 2 dKO mice assessed using antibody array technology

| Mediator | Function | Fold change |
|---|---|---|
| Chemokine ligand 24 (Eotaxin-2) | Chemotactic factor | 3.4 |
| Chemokine ligand 1 (KC) | Immune response | 6.9 |
| Tumor necrosis factor (TNF-α) | Inflammation | 15.7 |
| Interleukin-12 (IL-12p70) | Inflammatory cytokine inducer | 2.3 |

Adapted from [17]

biomarker discovery and disease/disorder characterization are discussed in detail in other respective parts of this chapter.

### *1.2.2 Pathogenic biological processes—Clinical applications*

The application of biomarkers to diagnose a disease or provide a prognosis for a patient is perhaps antibody microarray's primary utility in the field of medicine. Without biomarkers that can reproducibly, accurately, and rapidly determine a medical disorder or human disease, doctors are often limited and ineffective at prescribing treatment regimens or minimizing suffering. In a clinical setting, biomarkers can be used for early diagnosis, disease classification and stratification, prognosis, patient monitoring, and personalized medicine.

Clinical relevance is outlined well in a publication on the role of creatinine levels and the existence of acute kidney injury (AKI). However, creatinine levels can fluctuate dramatically during deterioration in kidney function and thus are not optimal indicators of AKI, or are of limited use. In perhaps one of the most comprehensive reviews regarding the application of biomarkers to diagnose a medical condition, researchers at the Clinical Epidemiology Research Center in West Haven, Connecticut delineated a panel of biomarkers for the diagnosis of AKI [4]. This was a 7-year study range from MEDLINE and EMBASE pertaining to AKI serum and urinary biomarkers, looking over 25 meritable manuscripts. These identified that three key factors, serum cystatin C, urine interleukin-18 (IL-18), and urine kidney injury molecule-1 (KIM-1), were capable of differential AKI diagnosis, and other markers correlated strongly with early onset AKI and even AKI-related morbidity. Interestingly, as discussed below, these markers are now detectable rapidly, efficiently, and cost-effectively through the application of an AKI antibody array platform, or other antibody arrays now on the market.

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### 1.2.3 Drug toxicity

A great deal of drug development depends upon the monitoring of drug prototype toxicity as it applies at the cellular level and even at the macromolecular level of the entire organism. Thus, cohorts of biomarkers have now been developed for the *in vitro* and *in vivo* testing of drug toxicity. An example of this is the combination of biomarkers for the early detection of drug nephrotoxicity discovered by the company Compugen. In collaboration with the pharmaceutical company Teva, Compugen identified a signature panel of four biomarkers which are able to predict drug-related nephrotoxicity in rodents (http://cgen.com).

### 1.2.4 Drug efficacy

Biomarkers may also act as valuable entities for the monitoring of a drug's efficacy in preclinical research, during clinical trials and even in patients undergoing active treatment. Metabolites are especially powerful as biomarkers in this sense as they may act as global measurements of changes in an individual's metabolism in response to therapeutic intervention, as seen in studies of phospholipid metabolites as biomarkers of tumor growth [5]. Thus monitoring metabolite levels in response to tumor treatment may be an accurate and efficient method of determining the efficacy of drug treatments, as described recently for the antiangiogenic drug bevacizumab (BVZ) for the treatment of recurrent glioblastomas (rGBMs). In this study, ratios of membrane phospholipid metabolites and high energy phosphates were significantly different in rGBMs before and after BVZ administration, with increases seen in phosphoethanolamine/glyceroethanolamine (PEth/GPE) ratios after treatment [6]. This study confirms both the use of metabolites as biomarkers, as well as the utility of biomarkers to monitor and optimize drug responses.

## 1.3 Biomarker bottleneck

The past 20 years have thus seen a revolution in the discovery, characterization, and application of biomarkers in basic research, preclinical research, clinical trials, and clinical diagnostics. This revolution began with the advent of DNA sequencing technologies and the completion of the Human Genome Project. It is this wealth of information that has allowed for the rapid annotation and analysis of genes and the presence, absence, or modulation of corresponding transcripts levels. Both the discovery and application of nucleic acid-based biomarkers has now been automated in the form of gene microarray technology, and these "gene chips" has now allowed for

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the discovery and characterization of thousands of biomarkers in a single experiment. As discussed below, this solid-phase arrayed format for biomarker discovery and analysis has now been adapted in the context of biochemistry and, specifically, protein biomarkers. These new multiplex antibody arrays afford a chance to break the current biomarker bottleneck that has limited FDA approval of clinical diagnostic biomarkers to only a single approval per year from 1998 to 2006 [7]. This bottleneck likely arose from the lack of multiplexing technologies and/or methodologies for identifying new targets, but protein antibody arrays may mark the dawn of a new era of biomarker discovery, validation, and application.

## 1.4 Biomarker requirements

What are the requirements of a good biomarker? It depends upon the ultimate application, as every application may have different characterization goals. For example, a preliminary research experiment may require different qualitative and/or quantitative measures than for the prognosis or diagnosis of a patient's disease. In addition, clinically approved biomarkers must meet rigid regulatory requirements. In the United States, these include 510(k) premarket clearance or Premarket Approval (PMA) review and oversight. Yet some requirements for successful usage of a biomarker are universal, and apply not only to the biomarker itself but also to the sample in which it is present. Table 3 summarizes some of the more universal parameters that need to be met for preclinical research, drug discovery and validation, or patient diagnostics.

## 1.5 Protein biomarkers

It is important to emphasize that proteins are a rich source of biomarkers, and are currently used for the identification of countless medical indications. There are three primary ways in which the presence of proteins may reveal valuable information regarding a certain biological phenomenon or disease state. These include the presence of the protein itself, posttranslational modifications to the protein, or the elicitation of an immune response directed against the protein.

### *1.5.1 Protein expression*

First, protein expression levels provide unique insight into the existence of whole proteins in a system as they apply to marking a biological state. Alterations in normal levels of protein(s) may indicate that the system has gone

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**Table 3** Requirements for biomarker consideration and evaluation

| Biomarker parameter | Minimal requirement |
|---|---|
| Sample accessibility | Sample should be easily obtainable without harm to the patient or alteration of the experimental platform |
| Sensitivity | Changes in biomarker expression should be detected at low enough levels to discern differences in the normal versus altered biological process |
| Specificity | Changes in biomarker expression should be specific enough for the biological process being studied to clearly distinguish it from other processes |
| Accuracy | Changes in biomarker expression should be accurate enough to prevent mischaracterization of misdiagnosis of the biological process or medical disorder in 100% of the cases |
| Ease of analysis | Changes in biomarker expression should be obvious, providing a clear conclusion. This is also dependent upon the testing platform used |
| Rapid test result | Completion of biomarker characterization should take no longer than several days. This is also dependent upon the testing platform used |

awry. Levels of a protein may go up or down in response to a particular biological state or disease. C-reactive protein (CRP) is a good example of a key biomarker upregulated during, or in response to, inflammation. It is synthesized in the liver in response to factors secreted by both macrophages and adipocytes during an inflammatory response. CRP is particularly valuable as a biomarker as it gages the status of an inflammatory response which could be driven by countless diseases or medical disorders [8].

### 1.5.2 Posttranslational modification

Second, posttranslational modifications of proteins are very revealing with respect to the monitoring of specific biochemical pathways, from protein folding to glycosylation changes. Alzheimer's disease (AD) is characterized by the formation of lesions in the brain containing neurofibrillary tangles. Tau is a microtubule-associated protein which undergoes various posttranslational modifications upon aggregation into paired helical filaments in AD and other tauopathies, where phosphorylated tau is found in abnormal helical filaments. One of the classic hallmarks of AD is the presence of high levels

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of phosphorylated tau proteins present in cerebrospinal fluid (CSF). This has been confirmed in nine independent studies of phosphorylated tau as a biomarker of AD. In addition, each study confirmed the posttranslationally modified, phosphorylated tau as superior to unphosphorylated tau in the diagnosis of AD [9].

### 1.5.3 Autoantibody expression

Third, the expression of autoantibodies, which is an immune response directed against one or more of an individual's own proteins, provides unique information regarding the onset or aggravation of a disease or other medical disorder. Hepatitis C viral infection is well documented to elicit an acute autoimmune response. This response includes the generation of high levels of numerous autoantibodies including rheumatoid factor (RF), antinuclear antibody (ANA), cryoglobulin, and antismooth muscle antibody. Aside from obvious clinical manifestations of viral infection, such as arthritis, pulmonary fibrosis, and cryoglobulinemia, the presence of these antibodies in patient blood is highly indicative of a hepatitis C infection [10]. In relation to the latter two examples of posttranslational modification and autoantibody presence as biomarkers, proteomics technologies are the only efficient means of simultaneous marker detection.

From a clinical diagnostics perspective, proteins may provide a more accurate assessment of a patient's health, than detection of nucleic acids, for several reasons. First, genetic changes in the nucleotide sequence, tends to reflect the propensity or risk for a disease or disorder to occur, but not the presence of the disorder. Changes in gene expression levels as monitored at the mRNA level often correlate poorly with changes in protein levels [11,12]. Finally, in most instances changes in gene expression patterns do not consistently change the course or status of a disease, while protein presence and corresponding posttranslational modifications of proteins often do. Unlike gene mutations or mRNA transcript levels, protein existence and makeup is directly affected by various direct and indirect influences such as patient age, lifestyle, nutrition, exercise (or lack thereof), environmental factors, as well as genetics. In addition, in some instances the monitoring of autoantibody production or antibodies produced against invading pathogens is the only method of detecting such changes. Thus the monitoring of proteins and antibodies as biomarkers provides unique, rapid, and accurate insight into disease status or other biological processes.

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## 1.6 Protein biomarker discovery tools and technologies

The goal of diagnostic biomarkers requires these to be present in easily obtainable samples such as saliva, urine, serum, or plasma. Yet the characterization of protein biomarker levels present in these samples is not an easy task, and technologies must be available to satisfy the remaining criteria of sensitivity, specificity, ease of analysis, and rapidity. Numerous technologies have been developed over the years to characterize the presence of proteins and/or antibodies in biological samples. These technologies typically fall into one of several categories representing the biomarker discovery or clinical application process (Fig. 2). There are advantages and disadvantages to each which are discussed below.

### *1.6.1 HPLC coupled with MS*

High-performance liquid chromatography (HPLC) and mass spectrometry (MS) have been in existence for decades and each provides a truly unique and unbiased approach for the identification, validation, and application of biomarker characterization in a clinical setting. Liquid chromatography separates individual proteins from one another under relatively high pressure in the presence of a molecular sieve. After ionization of the isolates, MS measures the mass-to-charge ratio of the various proteins in the sample separated by HPLC. Tandem mass spectrometry involves multiple steps of MS selection, thereby significantly increasing signal-to-noise ratios for each analyte

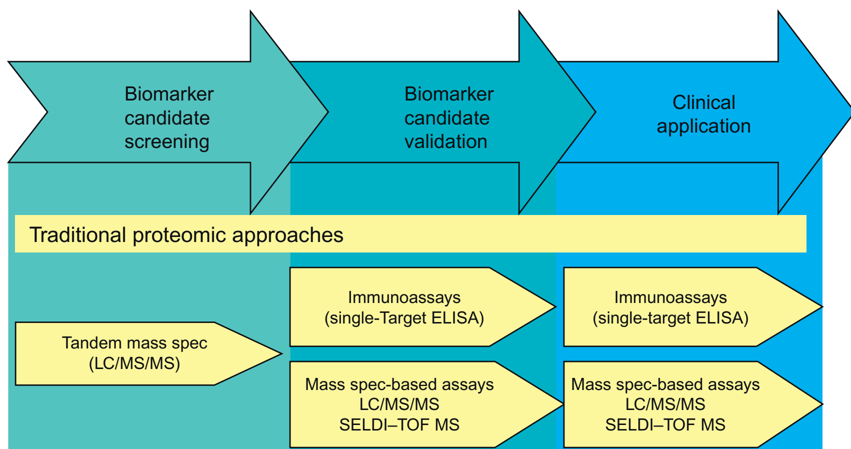


**Figure 2** Categorization of biomarker characterization technologies.

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present in the sample. Each of these methods for protein detection is considered to be "unbiased" due to the fact that they recognize peptide fragments representing a wide range of proteins. The implementation of LC and/or MS does not require a predetermination of what proteins might be present in a sample. Yet the sensitivity of LC and MS, ranging in the nanograms per ml, is relatively poor in comparison to other technologies such as the enzyme-linked immunosorbent assay (ELISA). ELISAs often detect analytes at concentrations as low as 1–2 pg per ml. In addition, LC and MS require expensive, specialized equipment along with highly trained personnel, resulting in a considerably high cost per sample tested.

#### *1.6.2 2D-polyacrylamide gel electrophoresis*

Although extremely low-throughput, polyacrylamide gel electrophoresis (PAGE) application to discover and characterize biomarkers merits discussion here. 2D-PAGE relies on a first dimension of protein separation based upon unique charge properties, followed by a second dimension of separation based upon protein size. The combination of these two phases of separation based upon unique characteristics of each protein results in individual spots of proteins segregated by charge/mass ratios. Detection of proteins can subsequently occur via Western blotting procedures or protein isolation from the gel followed by Edman degradation sequencing. While this is an elegant and long-standing method for either confirming the presence of known proteins in a biological sample, or discovering new proteins, it is considerably time consuming, low throughput, and requires stringent controls and expertise to be successful.

#### *1.6.3 Surface-enhanced laser desorption ionization—Time of flight*

Surface-enhanced laser desorption ionization—time of flight (SELDI–TOF) is an ionizing technology which enhances the ability to detect proteins using an initial surface separation step, followed by ionization and time-of-flight mass spectrometry detection. This technology was commercialized by Ciphergen Biosystems and the Protein Chip system now available from Bio-Rad. Although the sensitivity of the system is superior to LC/MS, it also has high-associated equipment and personnel costs. Perhaps the most significant disadvantages to all of these systems, however, is the inherent low-throughput nature, and the additional effort required to identify the unknown sample peak. Although elegant in concept, each of these systems can only detect one or relatively few proteins in a single experiment. Hence, relatively low-cost, high-throughput technologies, and platforms

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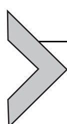
are needed for biochemistry-based biomarker discovery, characterization, and validation.

All of the above biomarker discovery and characterization platforms have unique attributes which make them valuable for certain applications, but each is hindered by disadvantages such as equipment and labor cost, time consumption, or limitation on high-throughput capacity. A technology is therefore needed that is reasonably priced, rapid, sensitive, specific, and does not require extremely specialized and costly equipment. Antibody arrays fulfill each of these requirements and more, and the remaining sections of this chapter will focus on the advantages and utility of antibody arrays in biomarker discovery and analysis.



## 2. ANTIBODY ARRAY PLATFORMS AND UTILITY

Antibody arrays are now widely recognized as a reliable and robust methodology for mining complex proteomes from numerous sample types and for multiple purposes. In addition to eliminating the need for separation and depletion techniques required by other protein identification assays, the high sensitivity of antibody probes allows for high-throughput and high-specificity detection at picomolar or even femtomolar quantities of target protein, all in a simultaneous multiplexed target platform.

Planar antibody microarrays consist of a large number of regularly arranged, discrete spots of capture antibodies which are applied to a solid support using spotting robots. The development and processing of antibody arrays are generally executed in the following sequence: research and developmental production and characterization of capture reagents, printing of capture reagents onto an appropriate solid support (nitrocellulose membrane, glass slide, etc.), sample incubation, detection of bound antigens (via chemiluminescence or fluorophore), and finally, data analysis. The binding of antigens to the antibody spots may be quantified using a secondary-specific detection antibody, a prelabeled sample (e.g., biotinylated), or with other specific molecules to the target protein. The identities of the bound antigens are then determined by the position of the signal on the array surface based on the map of the printed capture targets. Some common array platforms and designs can be seen in Fig. 3.

Like classical ELISA protocols, antibody arrays may be designed for fully quantitative output, wherein the array signals are standardized against a predetermined cocktail of antigens at known concentrations. Absent the standard cocktail, these arrays can measure the semi-quantitative signal

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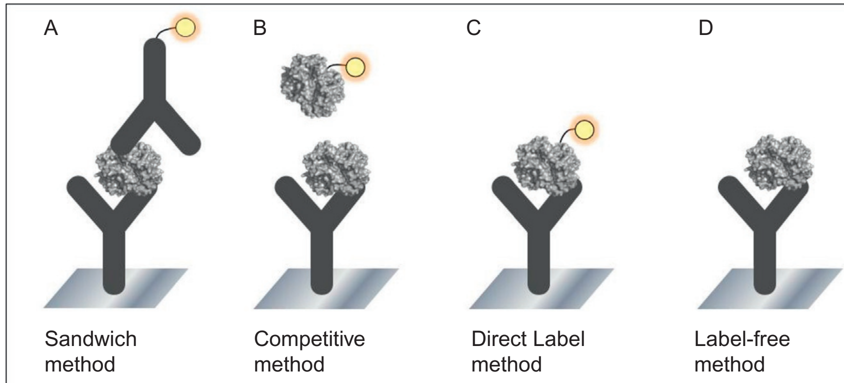


**Figure 3** Antibody array platforms. The sandwich method (A) requires an immobilized capture antibody, on a planar surface or a bead, and a labeled in-solution detection antibody. The competitive method (B) relies on competitive binding between the analyte and a labeled antigen. In the direct-label method (C), the label is attached to the analyte itself, while the label-free method (D) relies on an inherent property of the analyte (such as surface plasmon resonance) to produce a signal.

intensities that can be interpreted as relative fold-changes in protein expression levels (similarly to DNA microarrays), provided equal sample is loaded onto the original array. Sometimes both approaches are exploited in a biomarker study. Initially, the less costly semi-quantitative array serves to conduct an exploratory screen of the proteome, then candidate molecules from this prescreen may be subsequently included into a smaller quantitative array (multiplex ELISA) and applied for validation, sample stratification, or establishment of cut-off values. Together this provides a powerful tool to identify aberrant changes in protein expression from a number of different sample types, as well as an adaptable platform depending on the researcher's experimental needs.

Antibody arrays may be designed generally on one of three principles (Fig. 3). The sandwich-based immunoassay relies on a single capture antibody specific to the target protein, and a second detection antibody which is also specific to the target protein (termed antibody pairs). A second method uses a direct-labeling approach, whereby the sample is directly labeled with an agent that facilitates target detection prior to incubation with an array (e.g., biotin). Lastly, there is a label-free approach, which utilizes some other means inherent to the target molecule for detection. Each of these methods has various pros and cons for target protein detection, and these are discussed below.

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The sandwich-based method ELISA is the most preferred and commonly used antibody array, owing to antibody's excellent specificity and sensitivity. Part of this platform's preferred usage is owed in no small part to the reliability and acceptance of ELISA assays as a robust tool for protein level determination. As the name implies, a sandwich-based array utilizes matched antibody pairs: an immobilized (capture) antibody is printed onto a surface that "captures" the target molecule, and then a labeled (detection) antibody is added in to also bind the target molecule at a separate freely available epitope. The label on the detection antibody is then probed via fluorescence or chemiluminescence detection to measure the target molecule, where the signal intensity is related to the level of protein present in the sample (Fig. 4). Though highly robust, the drawback of this detection system is occasional cross-reactivity between the detection antibodies of combined sets of antibody pairs. Such cross-reactivity creates nonspecific binding of one antibody to another target protein, and such outcomes increase as the number of probed molecule antibody pairs increases. Thus each antibody pair must be carefully cross-checked against every other pair in the array panel, limiting the practical size of the array to approximately 100 antibody pairs [13]. This size limit can be overcome by using multiple sequentially designed panels to avoid non-compatible pairs, thereby allowing increased target molecules for detection across multiple arrays with only the need for more sample for the assay.

Alternatively, larger array panels may be constructed using the direct-labeling approach, where the antigens are labeled with fluorescent tags or biotin prior to capture, thus eliminating the need for antibody pairs [14]. This labeling approach offers many advantages, most significantly the lack

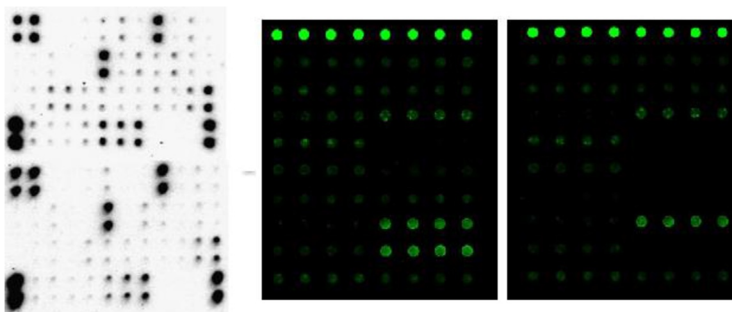

**Figure 4** Common antibody array platforms. Left, two membrane protein arrays with signals viewed by chemiluminescence detection. Right, two glass slide protein arrays with signals viewed via Cy3/green laser detection.

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of requiring an antibody pair, which for smaller proteins or novel proteins can be difficult to create and validate. Also, the removal of the secondary detection antibody essentially eliminates any potential antibody-to-antibody interactions which create cross-reactivity compatibility issues, allowing the array density to be scaled up to a theoretically unlimited size. The drawback of the direct-labeling format, however, is typically lower precision, exhibiting a coefficient of variation (CV) of 15–20% compared with a CV of 8–15% for sandwich-based multiplexed arrays. This approach is therefore limited to semi-quantitative measurement of protein levels. Additionally, the labeling process is generally nonspecifically targeted to certain chemical groups, which may alter the protein's functionality or mask epitopes the capture antibodies detect, creating both false negatives and false positives. If these types of labeling artifacts become significantly confounding, then smaller sandwich arrays, or a number of label-free detection methods should be considered.

Instead of attaching common labels to proteins (biotin, enzymes, or fluorescent tags), label-free methods rely on the detection of an inherent property of the target molecule. These technologies may include surface plasmon resonance (SPR), planar waveguide technology, electrochemical detection, quartz crystal microbalance (QCM), and microcantilevers [15,16,18]. Some label-free methods also have unique advantages; SPR for instance, not only measures concentration of a target protein, but also the strength of binding interactions, allowing the calculation of dissociation constants. SPR-based detection has been applied to antibody array chips for the detection of cancer-related cytokines in human serum, confirming such a tool for array usage [19]. Again, since a second specific antibody or protein is not used to confirm initial detection signal, the sensitivity is similarly limited as described for the labeled approach. However, with the lack of need for a secondary detection strategy, this method can prove fruitful with a properly designed experiment, and can yield additional information with regards to the target protein.

## 2.1 Antibody array signals

Signal levels for any detection method will often be the limiting factor in any analysis. However, there are ways to increase low signal events in order to improve the overall dynamic range of any array. This can be accomplished in one of two ways. First, a rolling circle amplification (RCA) method has been described which utilizes DNA amplification for the detection of

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antigen-bound antibodies [20]. In this system, the detection antibody is labeled with an oligonucleotide primer, which is then amplified in the presence of DNA polymerase and a synthetic DNA circle containing tandem copies of the primer sequence. The antibody is then detected after hybridization of a complementary fluorescent oligonucleotide. This amplification method results in greatly increased sensitivity, yet the technique is complicated, and costly protocols have hampered its widespread use, limiting its application in a high-throughput array system. Second, array signals can be amplified using a tyramide-based avidin/biotin signal amplification system. This approach has been demonstrated to be able to detect multiple cytokines at sub picogram levels, which could be extremely important for samples containing minute target protein, or for samples with limited volumes [21]. However, this amplification process sometimes increases the background signal, and would need to be tested to ensure the increased signal detection does not come at a cost of global background increase.

## 2.2 Antibody array platforms

How an array is built also affects its ability to perform the detection of targets in a sample. The solid supports used in the fabrication of planar protein antibody arrays include glass or plastic slides, standard 96-well ELISA plates, and nitrocellulose membranes. Membrane-based arrays typically have chemiluminescent readouts that are easily adapted to existing Western blot detection systems, making them an easy-to-use and low-cost option. Additionally, membrane arrays provide the comfort level of a commonly used research platform and technology the researcher is familiar with. The polymer-coated glass slide is another commonly used array support which provides the advantage of miniaturization, as the surface can accommodate minute capture antibody spots of less than 200 μm in diameter. This significantly reduces the array size and the sample volume consumption, as compared to the larger spots required for membrane formats [22]. Smaller array sizes also allows multiple arrays to be printed onto a single slide, making them increasingly suited for high-throughput analysis. Using current contact or noncontact array printers, a 25 mm × 75 mm glass chip can accommodate thousands of antibody spots. 96-well ELISA plates are another frequently used platform with array printers, with the printers spotting the bottom of each well with the capture antibody. This format provides the advantage of high-throughput and adaptability to ELISA-based automated workstations, which are common in laboratories already familiar with single-target

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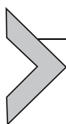
ELISA methods. The disadvantage of ELISA plate-based arrays is that the number of antibodies that can be accommodated by each well is currently only 16, given the size limitation of the physical spotting area within each well.

## 2.3 Antibody array signal detection

Fluorescence is by far the most commonly used readout system, offering high signal stability and wide dynamic signal range, and can be detected with common DNA microarray scanners or via flow cytometric detection equipment [23–26]. One platform of antibody arrays uses fluorescent detection of a bead-based suspension of antibodies involving the attachment of capture antibodies to microspheres coupled to combinations of fluorescent dyes. Here, flow cytometry techniques take advantage of differential levels of fluorophores on microspheres which are carefully calculated to correspond to a single antibody marker for detection. The main limitations to these bead-based system is the requirement of an often expensive flow cytometry machine for detection, but it does afford a similar fluorescent readout for target detection, without the planar support.

Overall, for detection of proteins, lipids, carbohydrates, and more, there are a variety of antibody array designs available for cutting edge biomarker research. These arrays are being used to discover new areas of biology for novel drug target development, to identify new cancer biomarkers for patient prognosis, and for noninvasive identification of treatment efficacy, to describe a few. Throughout the following sections, we will describe recent uses of antibody arrays in the area of cancer, immunology, ophthalmology, diagnostics, and asthma to name a few.



# 3. ANTIBODY ARRAYS IN DRUG TARGET DISCOVERY

The key steps for drug discovery are ordered by exploring new drug targets, revealing the mechanism of drug actions and screening biomarkers to predict drug treatment outcome. This methodology is described briefly in Fig. 5. Cytokines play an important role in a plethora of cellular and host pathways, ranging from signals involved in cellular growth and chemotactic signals which promote cellular recruitment to areas of infection, to apoptosis and tissue remodeling [27]. Alterations in cytokine profiles, or aberrant cytokine expression has been identified as a critical element in numerous common diseases, particularly with cancer, allergies, atherosclerosis, and immunological disorders [28]. These perturbations in cytokine signaling

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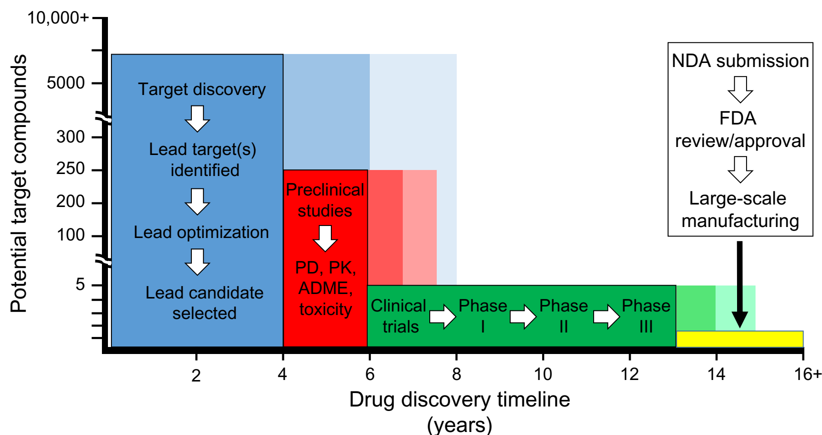


**Figure 5** Drug development methodology, from target discover to registration and launch. PD, pharmacodynamics; PK, pharmacokinetics; ADME, absorption, distribution, metabolism, and excretion; NDA, new drug application.

**Table 4** Cytokine alterations leading to cytokine drug targets

| Cytokine altered disease | Implicated cytokines | Cytokine-mediated therapies | Cytokine drug targets |
|---|---|---|---|
| Allergic response, colitis, HIV/AIDS, autoimmune diabetes, psoriasis, asthma, multiple sclerosis, Crohn's disease, ischemia, hypertension, graft rejection, COPD, and rheumatoid arthritis | EGF, Fas/FasL, IFN-α, IFN-γ, integrin alpha-4, interleukins IL-1, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IL-23, MCP-1, MIP-1α, NGF, RANTES, TNF-α, VEGF | Humanized monoclonal antibody therapy, chemical protein inhibitors, fusion proteins, receptor antagonists, recombinant proteins, and small molecule inhibitors | Natalizumab, Infliximab, Adalimumab, CDP571, PEG Fab, Lenercept, Antiferon, Lerdelimumab, B-N10, Etanercept |

Adapted from [17]

and/or expression provide prime targets for drug discovery and development, as is currently being done by a number of pharmaceutical companies for cytokine-associated diseases (Table 4). Cytokine-targeted therapies most often include small molecule inhibitors, monoclonal antibody (mAb) therapies, and recombinant proteins as a means to normalize the levels of the targeted cytokine [29–31]. However, identification of important cytokines in these diseases remains only as efficient as the techniques used to study the disease model system itself.

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Conventional single-target assays (ELISA, Western blot) are great for confirmation of a known theory, or following up after an initial screen, but can be expensive, time consuming, and sample limiting for biomarker discovery. While some disease states may occur when only a single cytokine is altered, more often it is the interplay between several cytokines, thus studying these diseases requires a more global perspective of cytokines. Additionally, with only a single cytokine being evaluated, the researcher must know the target of interest, or have narrowed the list down sufficiently to determine and evaluate any biomarker of importance in the studied disease state. If their model does not rely on a single target for detection or treatment, the techniques required for detection of these biomarkers are further limited by using a single-target method. As such, high-throughput multiplex techniques need to be incorporated, especially in the early phases of drug discovery to assess cytokine interactions on such a macroscale. Such a broad initial view can narrow the field of candidate markers, increasing efficiency, decreasing costs, and requiring less initial sample. The advent of cytokine antibody arrays in the early 2000s have provided this exact platform, which can be exploited to view cytokines on a global scale [17,32]. This visualization of a more complete sample cytokine profile can facilitate discovery of unexpected disease pathways, identify biomarkers that intimate target drug efficacy, and also identify biomarkers of potential disease resistance.

These cooperative and redundant cytokine pathways are probably best described in cancers, where the single drug target/pathway approach is hindered by a cancer cells ease in escaping a single drug targeted pathway. A great example of this complex interaction can be found in a recent publication by Torres *et al.* [33]. This group was studying potential biomarkers of pancreatic cancer, and how chemotherapies affect those biomarkers in pancreatic cancer patients *in vivo*. Due to the often late diagnosis of pancreatic ductal adenocarcinoma (PDAC), coupled with the significant chemoresistance, early diagnosis, and treatment options are limited for a disease with a 5% 5-year survival rate. In an attempt to devise an early diagnostic tool for disease screening, 39 patients were enrolled in a study on serum biomarkers of disease. Serum samples from 12 healthy volunteers, 14 untreated PDAC patients, and 13 PDAC patients undergoing chemotherapy with gemcitabine + erlotinib were probed across a cytokine array recognizing 507 proteins. This array identified five cytokines that were significantly overexpressed in untreated PDAC patients compared to healthy controls. These five cytokines (fibroblast growth factor 10 (FGF-10), CXCL11, oncostatin M (OSM), osteoactivin, and stem cell factor (SCF))

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showed an individual specificity range of roughly 75%, but when combined together, this specificity increased to over 84%. Overall this provided a unique biomarker subpanel for potential pancreatic cancer screening in serum.

A separate finding in this same study was equally interesting, as the group compared the 14 untreated PDAC patient serum samples to the 13 PDAC patients undergoing combined chemotherapy (gemcitabine + erlotinib). Interestingly patients having undergone chemotherapy for 2 weeks stabilized the expression of SCF and chordin-like factor 2. Chemotherapy treatment also returned the levels of FGF-10 (and even reduced compared to healthy controls), increased the levels of CXCL11 and oncostatin M over healthy controls, and resulted in profound decreases in CD30 ligand and GDF-15. These changes, both between healthy patient samples and within treated and untreated groups, provide the foundation for drug discovery and a starting point for diagnostic screening and drug target development. For example, FGF-10 is a promoter of pancreatic organogenesis and has been linked with invasion of pancreatic cancer cells via induction of MMP1 [34,35]. SCF binds c-kit which promotes cellular proliferation and survival in c-KIT expressing cells, and has been shown to be elevated in PDAC patient serum as well as other cancer subsets [36–38]. Both of these might therefore prove to be prime drug targets for development, via targeted small molecule drugs to SCF on tumor cell surfaces, or antibodies directed at FGF-10 expressing tumor cells.

Another example of potential cancer drug targets elucidated through antibody arrays, is a recent study by Gest *et al.* involving two breast cancer cell lines [39]. Ras-related C3 protein (Rac3) is a Rho GTPase, a known regulator of cell growth, whose signaling results in downstream activation of NF-κB. Using an siRNA knockdown of Rac3, this group noted that only the invasive MDA-MB-231 cells responded with reduced aggressiveness as measured by decreased invasion, reduced adherence to collagen, and an increase in TNF-α-induced cell death. The noninvasive MCF-7 cell line was not affected by the Rac3 knockdown. Antibody array analysis revealed that the Rac3/NF-κB pathway could be involved in the secretion of matrix metalloproteinase (MMP)-9, IL-6, IL-8, and growth-regulated oncogene (GRO), while also potentially being involved in the TNF-α resistance. While the exact role for Rac3 remains to be elucidated, this finding has directed attention to new targets of interest in dealing with aggressive breast cancers, as well as for profiling other aggressive cancers that may utilize the same Rac3 pathway for malignancy.

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The availability of ever larger antibody arrays allows more and more breadth in biomarker and novel drug discovery. Using an antibody array against 507 cytokines Li *et al.* discovered novel biomarkers of pediatric osteosarcoma for drug development [40]. The antibody array identified three cytokines, CXCL4, CXCL6, and CXCL12 that were significantly elevated in pediatric osteosarcoma patients when compared to healthy controls in sample plasma. Of significant interest was the finding that when CXCL4 and CXCL6 were significantly found in patient plasma, the patient outcome correlation was negative. Since anticancer options for osteosarcoma patients at the time was chemotherapy alone, the proposition for targeting the CXCL4 and CXCL6 pathways may provide promise for future treatments. Additionally, using these markers as predictive tools may provide early evidence to begin more drastic treatment options sooner to improve patient outcome.

Vascular endothelial growth factor (VEGF) expression levels are upregulated in most tumor and tumor stoma cells, due to a number a tumor-specific conditions such as hypoxia, acidic pH, hypoglycemia, and several inducing cytokines. Additionally, tumor genetic and epigenetic changes further promote VEGF expression of conditions that favor its increased levels. VEGF binds to two receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1), and VEGFR-2 (KDR, Flk-1) [41]. It is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF. Additional growing evidence suggests that VEGFR-1 has significant roles in hematopoiesis and in the recruitment of monocytes and other bone marrow-derived cells that may home in on the tumor vasculature and promote angiogenesis [42–44].

Determining the efficacy and outcomes of drug and chemotherapies is another critical mechanism involved in the drug discovery and evaluation process. A group at MD Anderson studying antiangiogenesis treatments, via anti-VEGF therapy for glioblastoma tumors, noted that while the treatment was initially effective, tumor populations eventually escaped via reactivation of other pathways of angiogenesis [45]. This reactivation occurred alongside increased MMP2, MMP9, MMP12, and TIMP-1 expression. The group used an antibody array to decipher that this reactivation of angiogenesis was partially the result of increased expression of angiogenin, IL-1β, TGF-α, and TIMP-1 and TIMP2, all molecules with angiogenic and invasion potential. Given these results it was surmised that the strategy for anti-VEGF escape by these tumors might warrant treatments that target not only the VEGF pathway, but also the MMP pathway

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which the tumors appear to subvert in order to invade surrounding neurological tissues.

Similarly, Lin *et al.* was working to identify various cellular markers that contributed to breast cancer invasiveness and tumorigenic properties [46]. This group evaluated several breast cancer cell lines for the secretion of 35 cytokines, and identified that IL-8 expression was significantly elevated in cells expressing less estrogen receptor (ER), cells of increased metastatic potential, and of known vimentin status. This correlation between IL-8 and ER suggested an interesting relationship between these two markers, as neutralization of IL-8 inhibited the cells ability to promote angiogenesis and invasive properties. Likewise, treatment of the cells with excess ERα inhibited IL-8 expression. This suggests that ER and IL-8 levels may be important prognostic indicators of breast cancer disease. Additionally, drugs that target this pathway may be novel targets for future development.

Biomarker discovery for potential drug targets or evaluating drug efficacy is not limited entirely to cancer, as numerous immunological disorders involve cytokine abnormalities. Henoch–Schönlein purpura (HSP) is an autoimmune disease characterized by systemic vasculitis and resulting purpura (skin surface hemorrhages). Disease associated immunoglobulin A (IgA) complex deposition in the kidney glomeruli leads to glomerulonephritis complications, and this common renal involvement can lead to end-stage renal disease (ESRD). Using a 20 target antibody array, Chen *et al.* identified elevated cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) in the urine of HSP patients with renal involvement compared to those without renal involvement or healthy controls [47]. The elevation in cystatin C and NGAL were then confirmed by single target ELISA against patients without renal involvement, as well as against a control group of atopic dermatitis patients without vasculitis. While the study group was small, and follow-on studies are warranted with respect to how the markers change as the disease subsides, such an early and noninvasive diagnostic panel is a critical finding in order to limit early renal damage, but also to treat progressed renal disease in HSP patients.

Given the immunological changes that occur during HIV infection, it is with no surprise that antibody arrays are being put to good use to derive novel cytokine data and produce new potential biomarkers for HIV infections. Sachdeva and Asthana identified three markers (IL-1β, VEGF, and EGF) that were expressed at higher levels in HIV discordant couples compared to concordant patients [48]. How this affects HIV infection, if at all, remains unclear, but these could be a subset of biomarkers for monitoring

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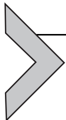
HIV infection in at-risk patients. Separate studies of HIV infection stages also correlated to expression levels of various cell surface cytokines in HIV infection as a goal of identifying the HIV disease stages. These studies identified 17 statistically differentially expressed proteins between CD4 and CD8 T cells, notably confirming several markers previously published with flow cytometry and also identifying 5 new ones [49]. A separate group used a large 274 protein target cytokine array to identify a link between 14 chemokine and cytokines expressed differently in viremic and aviremic states [50]. Additionally, a novel peptide array is under consideration in an HIV vaccine trial, where monitoring of gp120's V2 loop is critical for viral immunogenicity and subsequent protection from infection. It is being used to evaluate the overall humoral immune response to the virus, and has been integral in the trial [51]. Together, these findings support the use of antibody arrays in the pursuit of new novel targets of potential correlates of diagnostic value for cancer and other diseases when evaluating new drugs and treatment options.



## 4. ANTIBODY ARRAYS IN THERAPEUTIC BIOMARKER DISCOVERY

There is a long history of belief that antibodies could serve as "magic bullets" in the diagnosis and therapy of cancer. Although this has not completely come to fruition, and it is not without some controversy, these specific molecules are still widely appreciated as critical to the cancer community. Their use includes every aspect of cancer studies from biomarker evaluation, to diagnostics, to potential treatments, and prognostic indicators. Specific examples of biomarkers like carcinoembryonic antigen (CEA) as a marker for colon and other cancers, and discovery of the α-fetoprotein as a marker for hepatocellular cancer is particularly exciting for the field and lends credence to the hope of more biomarkers to come [52–54].

The mechanism of tumor cell killing for cancer treatments can be generalized to one of multiple kinds of mechanistic pathways: direct antibody-mediated cell death, immune-mediated cell killing mechanisms (via IFN-γ, TNF-α, etc.), and specific effects of antibodies on tumor vasculature and stroma. These have been well defined recently by Scott *et al.* to ascribe various therapeutic agents against the corresponding tumors, and these have been briefly summarized in Table 4 [55]. Some of the many antigens that have been identified as suitable targets for antibody-based therapies in cancer include VEGF, EGFRvIII, CD20, CD30, and ALK [56]. Although

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antibody-based therapy has enormous potential, we are just beginning to scratch the surface of antibody-mediated therapies, and to explore the true potential of these molecules in the control and treatment in cancer (Table 5).

General tumor angiogenesis theory, postulated by Folkman, indicates that tumor growth must rely on sufficient nutrient and oxygen supplies from the local environment. The source of this fresh and constant nutrient supply is provided for by tumor-localized angiogenesis; the creation of new blood vessels. Interestingly, VEGF is a potent angiogenic growth factor often found in high concentrations in the tumor microenvironment [57,58]. VEGF can therefore be seen as, and has been utilized as, both a diagnostic indicator, as well as a treatment option for cancer patients. Antibody-mediated blockade of VEGF, or its receptor is being explored in numerous cancer treatments to prevent tumors from generating the blood supply needed for unhindered expansion. Also, detection and quantitative measurements may serve as positive or negative prognostic correlates, or as a means to measure other drug therapies associated with this or similar angiogenic pathways.

Using a quantitative antibody array, Casanovas *et al.* looked to quantitative the level of a variety of proangiogenic factors, including VEGF and FGF-2, in the regulation of angiogenesis and tumor growth [59]. Specifically, the group was interested in understanding the role of VEGF receptors in angiogenesis as it relates to tumor growth and initial tumor establishment. Their results indeed found that VEGFR-2 is necessary for initial tumor

**Table 5** Monoclonal antibodies targeted at cancer antigens

| Classification group | Example antigens | Monoclonal antibody therapies | Cancer models |
|---|---|---|---|
| Hematopoietic antigens | CD20 | Rituximab, tositumomab | Non-Hodgkin's lymphoma |
| Glycoproteins | CEA | Labetuzumab | Breast, colon, and lung tumors |
| Glycolipids | Gangliosides | 3F8 and KW-2871 | Epithelial tumors |
| Angiogenesis | VEGF | Bevacizumab | Tumor vasculature |
| Growth receptors | EGFR | Cetuximab, panitumumab | Lung and colorectal tumors |
| ECM proteins | Fibroblast activation protein | Sibrotuzumab | Colorectal cancer |

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growth and persistent angiogenesis, as blockade of VEGFR-2 (but not VEGFR-1), resulted in limited tumor establishment. Eventually, however, VEGFR-2 antibody blockade resulted in tumor escape, with VEGFR-2 resistance occurring alongside an angiogenic switch to FGF family members for recruitment of new blood vessels to the tumor surface. Together this identified the reasons why single-target therapies may be of limited long-term use, but also uncovered a potential dual treatment, or next in line treatment option for potential follow-up studies.

Another antibody array from Bao *et al*. illustrated that the expressed levels of VEGF in stem cell-like glioma cells (SCLGC) is consistently higher than non-SCLGC under normal and hypoxic conditions [60]. These VEGF levels were sufficient for the induction of cell migration by endothelial cells and tube formation in an *in vitro* cell culture model of angiogenesis. These effects were not seen in control non-SCLGC conditioned media samples, suggesting a potentially novel target for this cancer. Also, since SCLGC can be a crucial source of several other key angiogenic factors, targeting these elevated proangiogenic factors from stem cell-like tumor populations may be critical for patient therapy at limiting or slowing disease progression.

Abajo *et al*. used a larger 44 target angiogenesis antibody array in order to measure the secreted factors from a large selection of colorectal cancer (CRC) cell lines [61]. Here, they found that unlike other studies, VEGF was not associated with metastatic cancer cell lines according to the initial antibody array, and later confirmed with a standardized competition ELISA. However, a large panel of other markers was found that could serve as indicators of metastatic cancer cell lines including angiogenin-2, MMP-1, IL-2, and IL-1α, when compared to primary CRC cell lines. Additionally, hypoxic conditions did induce VEGF expression in metastatic cell lines, but not to the level seen in primary CRC cell lines, suggesting that VEGF targeting at the metastatic stages of CRC may be of limited utility, while early detection of CRC may afford VEGF blockade as a viable means of treatment.

PDAC shows one of the worst mortality rates among common malignancies, with only 4% surviving up to 5 years after diagnosis. This is partially due to the often diagnosis of disease, and thus extremely delayed treatment, as well as due to the general chemoresistance nature of the tumor. In fact, to date there are no effective therapies available for patient treatment. Additionally, there are also no effective serological diagnostic tools, further limiting the identification of the tumor to later disease states, and inherently limiting patient options. At present, cancer antigen 19-9 (CA19-9) remains the only viable serological marker of PDAC, but its usage for diagnostic

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purposes are limited for a variety of reasons. Analyzing serum proteins after co- and/or posttranslation modifications has become an important field for biomarker discovery and analysis that expands the potential pool of diagnostic and therapeutic candidates. Of particular interest is the study of glycoprotein changes on the cell surface that have been highly associated with several forms of cancer.

A group led by Lubman at the University of Michigan used a unique antibody:lectin array to detect glycosylation changes on four proteins associated with pancreatic cancer [62]. Target antibodies were printed on an array surface, sample was bound, and then biotinylated lectins specific for known glycan moieties were used to assess individual glycosylation changes on target proteins in pancreatic cancer patient serum compared to controls. This unique strategy allowed for high-throughput lectin screening in the detection of differential glycosylation patterns in serum samples, and had excellent reproducibility during the researcher's studies. This array made it possible to discriminate cancer from the other disease groups and normal samples, with high sensitivity and specificity. The specificity came primarily from the levels of the alpha-1beta glycoproteins binding to the lectin SNA (Sambucus nigra bark lectin), which was increased by 69% in the cancer sample compared to the other noncancer groups. This confirmed the potential of these lection/glycosylation arrays as a biomarker discovery platform.

Therapeutic biomarkers may be most prevalently studied in the cancer field, but these markers for therapy also exist in other disease states where valuable treatment options and prognostic measure can be determined. Etanercept is a TNF inhibitor molecule approved for the use in treatment of rheumatoid arthritis (RA). It is also used to treat juvenile idiopathic arthritis (JIA) when NSAIDs are ineffective at limiting pathology. A recent study by Chen *et al.* in Taiwan used an inflammatory antibody array to monitor the plasma of JIA patients on Etanercept, and noted a significantly improved regulation of several inflammatory molecules (IFN-γ, IL-2, GRO, and MCP-1) [63]. This confirms the potential of Etanercept in treating JIA patients, but also suggests large-scale antibody arrays can prescreen patients for markers that warrant future drug analysis.

Etanercept is also used in severe cases of ankylosing spondylitis, a disease of the axial skeleton caused, at least in part, by chronic inflammation due to idiopathic reasons. Given Etanercept's antiinflammatory potential, a recent study of Korean patients receiving Enteracept treatment hoped to evaluate the drug treatment outcome. Twelve weeks post treatment, vast improvement in disease score and function index were identified, and these

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improvement correlated with cytokine expression changes among several cytokines measured [64]. Most notably, MMP-3 levels, as it relates to serum CRP and erythrocyte sedimentation rate, were identified as a potential biomarker for monitoring Etanercept efficacy in this chronic disease.

RA patients are also treated generally with methotrexate, an antiinflammatory steroidal therapy that is effective in up to 70% of RA patients. To study why some patients are unresponsive to methotrexate therapy, Dhir *et al.* monitored the levels of multiple cytokines in a cohort of RA patients undergoing various treatments. Looking at myeloid progenitor inhibitory factor 1 (MPIF-1), the authors noted that patients with a lower baseline level of MPIF-1 at the start of therapy, similar to healthy control levels of MPIF-1, are much more likely to respond to methotrexate [65]. This could provide an important diagnostic tool for evaluating therapeutical intervention in screening RA patients, helping to discriminate patients needing a different treatment for their RA, and those that are most likely to respond.

Biomarkers have even been evaluated for determination of outcomes in stroke patients. A recent study of a large cohort of ischemic stroke patients was undertaken by Navarro-Sobrino *et al.* to study the role of angiogenesis in stroke recovery patients, and how various biomarkers might help evaluate the subsequent patient outcome [66]. They kinetically measured plasma biomarkers in control versus stroke patients undergoing tissue plasminogen activator therapy. Interestingly, they noted that an early balance of proangiogenic cytokines with their angiogenic inhibitors was mostly associated with patients who would experience only short-term neurological deficit. However, those patients who saw increased plasma levels of antiangiogenic inhibitors, namely endostatin, were associated with an increased risk of worsening neurological disease. Evaluation of endostatin in patient plasma may therefore provide for a unique biomarker for drug or therapeutical intervention in stroke patients, and help direct doctors to consider alternative measures to treat at-risk patients.

A number of potential diseases can be evaluated with antibody arrays to determine potentially novel drug targets. As you can see outlined in Table 6, this strategy and others have allowed for the development of several new novel cancer biomarkers recently. From measuring changes in patient samples during treatment, to retrospective studies of patients who have recovered from disease or succumb to disease, antibody arrays can be used to simultaneously measure multiple cytokines in a high-throughput manner. New cytokine targets available in planar multiplexed arrays are being added

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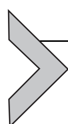
**Table 6** New therapeutic biomarkers being utilized to fight cancer

| **Therapeutic biomarkers** | **Tumor types** |
|---|---|
| TPX2 | Non-Hodgkin's lymphoma and prostate cancer |
| High-density lipoprotein (HDL) | Breast cancer |
| FGF18 | Ovarian cancer, colorectal carcinoma |
| Ubc9 | Melanoma, breast cancer |
| USP15 | Breast, ovarian, and glioma tumors |
| C-reactive protein (CRP) | Colorectal cancer |
| Matrix metalloproteinases (MMPs) | Pancreatic, lung, colorectal, ovarian, and prostate tumors |
| STAT3 | Breast cancer, renal cell carcinoma |
| GOLPH3 | Breast, esophageal squamous cell, prostate renal cell tumors |
| Aurora B | Nonsmall cell lung carcinoma |
| Telomerase | Lung, bladder, and breast cancer |
| Glycogen synthase kinase-3β (GSK-3β) | Glioma, bladder, and colorectal carcinoma |

every day, and new technologies which also are capable of detection protein modifications like phosphorylation and glycosylation, will further enhance the biomarker tool belt.



## 5. CYTOKINE ANTIBODY ARRAYS IN ASTHMA BIOMARKER DISCOVERY

Asthma is a disease characterized by the remodeling of airway epithelium, which permanently obstructs airflow due to underlying chronic inflammation of the small and medium airways. This underlying inflammation is exacerbated following allergen and irritant reexposure, due to the hyper-responsive bronchoconstriction of the airways, ultimately leading to an asthma attack. Diagnosis and prognosis of asthma, and a related airway condition, chronic obstructive pulmonary disease (COPD), are limited due to the lack of serological biomarkers of disease. Recently, two groups, Wadsworth *et al.* and Patil *et al.*, used biomarker antibody arrays to study and evaluate potential disease biomarkers, and have proposed several

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proteins as potential disease biomarkers (ECP, CCL18, TARC, HC gp-39, fibrinogen, and FGF, HGF, and SCGFβ, respectively) [67,68]. Because of the pathophysiological importance of inflammation in both asthma and COPD, inflammatory-focused antibody arrays have been employed to evaluate the protein levels in blood, sputum, and exhaled breath condensates in affected individuals, largely with good success.

One group of investigators studied the cytokine expression profile in patient sputum, comparing asthmatic and nonasthmatic control patients using an antibody arrays. Asthmatic patient sputum displayed significantly elevated levels GROα, Eotaxin-2, and PARC (CXCL1, CCL24, and CCL18, respectively) [69]. Interestingly, the levels of PARC in the patient sputum were significantly correlated to the numbers of eosinophils in the sputum sample, a known diagnostic factor associated with asthmatic patients. This suggests a novel diagnostic tool for asthmatic patients with a more non-invasive sample type required, and also outlines a potentially novel role and therapeutical target for PARC during asthmatic disease.

Exhaled breath condensates are a unique patient sample type that has recently been explored to measure inflammation during asthmatic symptoms. As such, there was no surprise that inflammatory markers were expressed at higher levels in asthma patients. However, what Matsunaga *et al.* noticed using an inflammatory multiplex array was that some inflammatory markers were significantly correlated to asthmatic patient symptoms [70]. By using the standard measures of patient symptoms, including forced expiratory volume in 1 s ($FEV_1$) and hyper-responsiveness to irritant challenge, the authors noted a surprising correlation between RANTES (CCL5) with $FEV_1$, and TNF-α and TGF-β1 expression with airway hyper-responsiveness. Additionally, the more severe a patient's symptoms, the greater the correlation with the cytokines was detected, suggesting a cytokine:symptom linear correlation. Subsequent experiments confirmed that the correlation seen was not due to cytokine contamination from patient saliva, suggesting a novel use of antibody arrays and the discovery of a noninvasive patient procedure [71].

Even more exciting is a recent study comparing exhaled breathe condensate (EBC) and bronchoalveolar lavage fluid (BALF). BALF lavage from patients is a very invasive procedure requiring flushing of the respiratory tract with fluids. Nakamura *et al.* used an antibody array to identify several cytokines in EBC that were correlated to cytokines in the BALF, including TNF-α and RANTES [72]. They also found that these correlated cytokines were also significantly related to the percentage of lymphocytes in the BALF,

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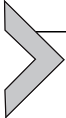
a critical diagnostic measure for patients with lung disease and/or in lung transplant patients. This identifies an interesting usage for EBCs in the study of patients requiring BALF lavage, possibly removing the need for the invasive procedure.

In a follow-up experiment by Matusunaga *et al.*, antibody arrays were used to compare how corticosteroid therapy altered cytokine responses during asthma attack compared to cytokine levels prior to treatment (baseline) [73]. EBC cytokine profiles showed that increased levels of IL-4 and RANTES coupled with decreased levels of IL-10 present during the initiation of corticosteroids were more likely to respond to therapy, as demonstrated by improved $FEV_1$. This improvement in overall airway obstruction correlated with subsequent decreased expression of IL-4 and RANTES in EBC samples. These studies imply that the usage of IL-4 and RANTES as predictive biomarkers for the treatment and success of steroidal therapy treatment may be of great use to doctors. Such a dual threat option for cytokine antibody arrays is the hallmark of biomarker identification and analysis, as it allows the tool to be used not only against the disease in both a predictive and prognostic manner, but also as a tool for predict treatment response and disease progression.



## 6. CYTOKINE ANTIBODY ARRAYS IN NEUROLOGICAL & NEURODEGENERATIVE DISEASE BIOMARKER DISCOVERY

Even with our current wealth of knowledge about neurological and neurodegenerative disease, numerous aspects of disease development, outcomes, and treatments remain out of reach. These processes are likely complex interactions between neural cell types, immunologic populations, cytokines, and chemokines, together implying a need for an assay that can globally monitor local and systemic protein changes and patterns. Cytokine antibody arrays afford such a global snapshot of protein expression and expression levels, and will likely be required for biomarker discovery given this multifaceted nature of neurological diseases.

The landmark study by Wyss-Coray at Stanford fully illustrates the potential for antibody-based arrays in the identification of disease biomarkers. Using plasma samples from 43 previously diagnosed AD patients, compared to 40 matched nondemented controls (NDC), the authors discovered consistent and salient differences among the 120 cytokines profiled in the patient's plasma [74]. Eighteen plasma biomarkers were identified

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via cross-validation, thus creating a matrix able to classify patients as having AD. After the initial characterization of this biomarker matrix, it was tested against a sample set of 42 AD patients, 11 with other types of dementia (OD) and 39 NDC. Cytokines measure of the same 120 cytokines was again performed, and a prediction analysis of microarray (PAM) scoring matrix was used to classify the patients using the 18 suggested markers. This method correctly identified the clinical diagnosis of these patients with an overall accuracy of 89% (90% agreement for those with AD and 88% agreement for non-AD), confirming its potential use as a biomarker test for AD.

The next set of testing data, however, was more important, as the authors tested patient plasma samples from 47 presymptomatic patients (patients with mild cognitive impairment (MCI) that had subsequently been followed longitudinally). MCI is a known early warning sign for potential AD development, and while not always progressing to AD, is an important diagnostic indicator. Some patients over time had been diagnosed with AD, while others had been diagnosed with other types of neurological disease, creating an excellent test set to probe with the biomarker array. Incredibly, the same 18 biomarkers using the PAM scoring system correctly predicted the neurological outcome. The authors were able to predict which patients were ultimately diagnosed with AD with 81% accuracy (91% agreement with AD patients and 72% for non-AD patients). Moreover, the plasma samples were taken up to 6 years before clinical diagnosis with AD, suggesting this 18-marker panel may be an incredibly valuable early screening test for AD. Additionally, the test may be able to rule out AD and recommend other neurological treatment for patients with MCI. While recent follow-up studies have had trouble confirming these 18 markers in their settings, it none-the-less describes the potential for antibody arrays in the generation of potentially critical diagnostic blood tests, but also describes the overall strategy with which to tackle disease screening and future biomarker investigations [75].

Additionally, recent studies have adopted a technique of evaluating biomarkers across studies rather than focusing on individual studies to validate blood-based signatures of disease [76,77]. Doecke *et al.* tested a biomarker panel of 174 targets, compiled from several array type options, to study a cohort of 754 diseased patients compared to 207 control health patients [76]. Their data were also used to cross-compare against a second cohort of 112 AD patients and 58 healthy patients. Their biomarker study indicated that an 18-biomarker panel produced a sensitivity to disease detection of 85%. This panel could be further reduced to include only eight biomarkers (cortisol,

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IGFBP2, PPY, IL-17, VCAM1, β2 microglobulin, epidermal growth factor receptor (EGFR), and CEA) with only a 2% reduction in sensitivity. Studies like this one have provided additional evidence for the potential presence of clinically and diagnostically important plasma biomarkers for AD, and further confirmed the promise of arrays for biomarker discovery.

Dr. Wyss-Coray's group has continued to use antibody arrays and data analysis methods to search for other markers of neurodegenerative disease. This group recently evaluated 776 potential biomarkers for screening sporadic AD patient plasma. Compared to healthy controls, a Significance Analysis of Microarray (SAM) algorithm identified 42 proteins that were differentially expressed, including commonly associated proteins like amyloid precursor protein (APP) and apo-lipoprotein E (ApoE). Additionally, connectivity and pathway analysis revealed further promising biomarker connections, whereby a common signaling pathway related to TNF-α, TNF-β or angiogenesis seems to be at work (personal correspondence). And, using a different algorithm, elastic net regression (eNet), this group identified additional potential plasma biomarkers, which together with their previous data and others within the field, could prove extremely valuable in the hunt for diagnostic and drug target biomarkers.

Exploring the molecular basis for neurological disease is another critical area where antibody arrays can facilitate the prognosis, diagnosis, and even treatment. Such examples can be seen with the use of antibody arrays in the search for the molecular basis for the role of neuroinflammation in autism. The autistic spectrum describes a broad range of neurological developmental disorders describing patients who display impaired social interaction, repetitive behavior, or difficulties in cognitive development. The exact nature of autism spectrum development is still unknown, but several recent studies have suggested a link to an inflammatory condition [78]. Vargas *et al.* recently investigated the potential correlation between autism symptoms and neuronal inflammation via a cytokine antibody array to screen for potential biomarkers in CSF and brain lysates [79]. Compared to age-matched controls, autism patients showed consistently higher expression of MCP-1 and TGF-β1 in several brain-sampled regions (among other cytokines of potential interest). This was the first seminal finding of the potentially impactful nature of neurological inflammation in the etiology of autism. In addition, this also provides for a potential treasure trove of target proteins which might mediate some role in disease development. While such a finding was certainly possible using standard ELISA or Western blot techniques, the user would have had to correctly hypothesize individual

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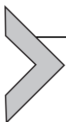
targets to evaluate in potentially precious sample types. However, antibody arrays allow interrogation of multiple cytokines simultaneously, providing an efficient platform for identification or discovery of a wealth of intriguing data for subsequent autism research.

Similar studies on various aspects of other neurological diseases have also been done from Parkinson's disease to prion-associated brain disease. Cytokine antibody arrays detected that senescent astrocytes (p16$^{INK4a}$+) are more prone to produce increased inflammatory molecules IL-6 and RANTES, cytokines known to be associated with chronic inflammation linked to age-related diseases like Parkinson's [80–82]. A related study attempted to identify cytokines in the blood of patients to discriminate healthy individuals from AD and Parkinson's patients. This used a small 22-multiplex cytokine array for their study, but were unable to determine an immunological cytokine pattern linked to disease, suggesting other sample types or target proteins need be evaluated to attempt to prescreen patients for these neurological disorders [83].

A recent unique use of multiplexed antibody arrays was done to determine any clinically significant immunological or cytokine changes that occur during scrapie infections in mice. Scrapie is a prion-associated neurodegenerative disease that is transmissible in goats and sheep, but is only clinically identifiable in late stages of disease. Outside of late-stage neurological disease, there are no diagnostic measures for transmissible spongiform encephalopathy infections (TSE), and currently no treatment to date exists for any TSE. This study used prion-infected mice and monitored the disease by measuring 62 target proteins in various tissues as well as in the animal's serum [84]. Interestingly, two markers, IL-10 and TIMP-1, were identified as consistently associated with prion infection, possibly suggesting not only important features of prion infection dynamics, but also of potential biomarkers of prion infection.



## 7. ANTIBODY ARRAYS IN IMMUNE-MEDIATED DISEASES

The immune system of an organism protects against disease by identifying and killing pathogens, as well as recognizing and preventing growth of tumor cells. The human immune system can be divided into the innate and adaptive branches. The innate immune system comprises physical barriers (such as skin and mucosa) and phagocytic cells (such as macrophages, granulocytes, dendritic cells, and natural killer cells) that recognize cellular stress or pathogen-associated patterns on the surface of invading microbes.

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Upon activation, this system can react immediately to release a variety of inflammatory cytokines and cellular apoptotic initiators that facilitates pathogen clearance, and also serves to initiate the adaptive immune response. While the innate immune system does not involve immunological memory, the adaptive immune system does through its cellular compliment of T- and B-lymphocytes, which create the cellular and humoral arms respectively. Following stimulation, the T cell compartment is activated to form $CD8^+$ cytotoxic effector cells and $CD4^+$ helper cells, while B-cells develops into antibody producing plasma cells. Normal immune functions depend on the ability of these immune cells to cross-talk and interact with each other in the appropriate manner. Dysregulation of the innate immune systems can cause autoinflammation, such as proinflammatory cytokine signaling abnormalities (like RA) or lack of normal bacterial sensing. Adaptive immune disorders instead promote the development of autoimmune diseases and can lead to tumor formation if the adaptive system fails to detect early cancer growth. Given lots of the immunologic cross-talk relies on cytokine and protein signaling, antibody arrays have been widely used to study all three categories of human immunological disorders: hypersensitivities, autoimmunity, and immunodeficiency.

IgE-mediated allergic diseases (such as food allergies and asthma) are type 1 hypersensitivity reactions which are among the most prevalent disease worldwide. While the component-resolved diagnosis (CRD) with arrayed recombinant or purified allergens showed promising results in the diagnosis of allergic diseases, the pathogenesis of allergy is still under evaluation [85]. Allergen-induced airway inflammation, which triggers T cell proliferation, is the main target for asthma serum biomarker research. During allergen stimulation, cytokines from primed TH2 $CD4^+$ T cells triggers B cell release of antigen-specific IgE. Allergen binding to the released IgE, and subsequent IgE binding to its receptor on the mast cells cause the release of histamine into the airway. Histamine release in turn triggers the rise of intracellular calcium, muscle contraction, and airway narrowing, hallmark symptoms of asthma. Currently, there is no single serological biomarker used in routine clinical asthma diagnosis and prognosis, though as described previously, several researchers are making quick progress [67,68].

Antibody arrays have also been used in the study of cell-mediated type IV hypersensitivities such as graft versus host disease (GVHD). GVHD is a common complication of allogeneic bone marrow transplantation in which donor's immune cells present in the transplanted marrow recognize the recipient as "foreign" and mount an immunologic attack. GVHD following

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allogeneic hematopoietic stem cell transplantation (HSCT) is the major cause of nonrelapse mortality. Pretransplant clinical or transplant characteristics have minimal ability to predict acute GVHD outcomes. Currently, GVHD diagnosis and disease prognosis rely entirely upon clinical symptoms and biopsy, as no laboratory test exist to predict the risk of developing GVHD, the responsiveness to any treatment, or patient survival. Substantial organ damage may have already occurred by the time a patient presents with GVHD symptoms, so a serological biomarker panel could therefore be a highly valuable and lifesaving diagnostic tool for conclusive GVHD diagnosis before the onset of symptoms. Using an array to simultaneously screen patient sera for 120 proteins, Paczesny *et al*. reported that a panel of 4 serum proteins (IL-2 Ra, TNFRI, IL-8, and HGF) can confirm the diagnosis of GVHD and provide prognostic information independent of GVHD severity [86]. To validate this GVHD biomarker panel, they randomly divided the samples into a training set (282 patients) and a validation set (142 patients). This validation confirmed the 4-protein biomarker panel for GVHD diagnosis with high-specificity, which provided the first evidence that these biomarkers are associated with GVHD clinical outcomes and prognosis, and serves as an important finding in the GVHD field. This was also extended recently by another group who blind tested a known sample set from GVHD patients and healthy controls, and validated a similar four-biomarker panel (HGF, TNFRI, ST2, and Elafin) with the aqueous two-phase multiplex ELISA technology [87]. These findings were further validated by single-target ELISA, showing the multiplex capability and similarity with single-target protein detection methods.

## 7.1 Autoimmune disorders

Autoimmune diseases affect 3–8% of the world population, and comprise a wide variety of systemic or organ-specific inflammatory diseases characterized by abnormal activation of immune cells that improperly target "self" tissues. The spectrum of autoimmune diseases includes RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS), systemic sclerosis (SS), type 1 diabetes (T1D), Psoriasis, and Crohn's disease (CD), among many others. Overall, the precise pathogenesis of the most of the autoimmune diseases is still poorly understood, and the disease diagnosis and classification still relies primarily on clinical examination combined with traditional laboratory testing and/or imaging studies. It is well appreciated, however, that inflammatory cytokines and chemokines appear to play a central role,

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findings which support antibody array focused studies for biomarker discovery [88].

SLE is a severe chronic autoimmune connective tissue disease, characterized by production of autoantibodies against a broad range of self-antigens including DNA, RNA, histones, and other nuclear components. The pathogenesis of SLE is a complex process, involving many pathogenic factors like deposition of autoantibodies in kidney glomeruli, chronic activation of complement and macrophages, cell proliferation, and dysregulated production of extracellular matrix proteins, proinflammatory cytokines, and/or chemokines. To date, genetic, epigenetic, and serological SLE biomarkers have been reported in the literature [89]. Historically, autoantibodies (e.g., anti-dsDNA, anti-C1q antibodies) are ubiquitously the most analyzed biomarkers for SLE diagnosis and disease management. However, more recently newer serum biomarkers have been identified for the disease with antibody array technology. Using a protein microarray platform, Bauer *et al.* identified a group of 30 interferon-regulated chemokines in SLE subjects, markers that could potentially be used not only for diagnostic purposed, but also for potential new treatment targets [90]. With a label-based approach, Carlsson *et al.* recently used a recombinant antibody array to identify a group of 60 immunoregulatory proteins in SLE patients [91]. Their data showed that panels of biomarkers can be effectively used to diagnose SLE and differentiate disease severity and activity. In a similar approach, Petersson *et al.* demonstrated that a 48-plex miniaturized planar recombinant antibody array can be adequately used to decipher SLE patients with healthy controls, all helping underscore the utility of arrays in biomarker discovery [92].

RA is another common autoimmune disease, affecting around 1% of the general population, and characterized by chronic inflammation and damage of the joints. The earlier treatment for RA begins, the more likely the patient can prevent irreversible damage of the joints. Clinically the detection of autoantibodies against RF and anticitrullinated peptide antibody (ACPA) are the routine biomarkers for RA diagnosis. Recent discoveries of other novel serological biomarkers to improve the early diagnosis of RA, as well as for disease stratification, have been studied and show promise. In recent years, cytokine antibody arrays have been heavily utilized to monitor the protein and cytokine expression profiles in RA patients and their response to various therapies. Kokkonen *et al.* noticed that cytokines and chemokines were upregulated before the onset of RA, and similarly Hueber *et al.* reported a panel of proinflammatory serum cytokines (IL-1β, IL-6, IL-13,

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IL-15, and TNF-α) for early RA detection [93,94]. Wright *et al*. reported that the RA synovial fluid had significantly elevated cytokine levels and three cytokines (IL-6, IL-2, and G-CSF) correlated well with the treatment of anti-TNF drug therapy [95]. Synovial fluid analysis in early onset RA patients lead Raza *et al*. to identify a profile of T-cell-derived cytokines that are significantly elevated when compared with established RA patients, indicating the active role of this cell type in disease pathogenesis, as well as uncovering potential treatment biomarkers for future studies [96]. In an RA etiological study, Hughes-Austin *et al*. demonstrated the association between RA-related autoantibodies and circulating cytokine and chemokine biomarkers associated with inflammation, and cytokine biomarkers have recently been extensively reviewed [97,98].

Given the complexity and multifactorial nature of RA pathogenesis, single biomarkers are likely insufficient for disease diagnosis and/or stratification, especially given some of the findings considered above. Through profiling autoantibodies, cytokines, and bone-turnover products in sera from 120 RA patients, Chandra *et al*. developed an automated, multiplex biomarker assay (IMPCT) for early RA diagnosis, with significantly high sensitivity and specificity [99]. A separate 12-plex blood test (Vectra DA) has also been developed to assess current RA disease activity, via measurement of 12 serum proteins, (IL-6, TNFRI, EGF, VEGFA, MMP-1, MMP-3, YKL-40, VCAM-1, CRP, SAA, Leptin, and Resistin) [100]. These serum markers were chosen to represent the biology of diverse pathways thought to be involved in RA pathogenesis. An algorithm is then applied to calculate a single Vectra DA score ranging from 1 to 100 that categorizes RA into low, moderate, or high disease activity. This small 12-biomarker test was independently validated in multicohort study involving more than 1700 patients to precisely measure RA disease activity, confirming its utility in the field [101].

MS is a rare chronic inflammatory demyelinating disease of central nervous system, where the insulating myelin sheath that protects nerve cells in the brain and spinal cord become degraded and lost over time. This damage disrupts the ability of parts of the nervous system to send signals rapidly down their axons to neighboring neurons, limiting cellular communication and resulting in a wide range of symptoms such as abnormal sensation, numbness, paralysis, blurred vision, muscle stiffness, cognitive dysfunction, and urinary problems. While the cause is not clear, the underlying mechanism is thought to center around immunological targeting of the myelin-producing cells, or via dysfunction of the myelin-producing cells. Cytokines

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are key factors in the regulation of inflammatory responses and may therefore reflect the underpinnings of the disease process in MS. Hagman *et al.* examined serum levels of cytokines, chemokines, and apoptotic molecules to profile different subtypes of MS and found that Fas and MIF were upregulated in MS patients [102]. Additionally, this group noted that TNF-α and MCP-1 were higher in primary progressive MS patients. Tumani *et al.* explored the patterns of TH1/TH2 cytokines in relapsing remitting MS patients to determine their relevance as potential biomarkers in response to glatiramer acetate (GA) therapy. They found the quotient of (IL-2 + IFN-γ)/(IL-4 + IL-10) was significantly elevated in patients with relapses compared with relapse-free patients [103]. In a similar vein, a smaller sample size was evaluated by Oreja-Guevara *et al.*, comparing the TH1/TH2 profile in relapsing remitting MS patients treated with GA or Natalizumab (NAT). Their research found that NAT treatment showed higher levels of TH1 cytokines compared to GA patients, while GA therapy promotes a superior TH2-biased antiinflammatory response, implying an association between drug and T cell compartment that is ultimately derived [104]. Using a large sample cohort, Martins *et al.* examined serum cytokine profiles from 833 MS patients and 117 healthy controls. Their results showed both proinflammatory TH1 cytokines and antiinflammatory TH2 were significantly increased in patients with MS compared with healthy control subjects, further indicating general cytokine heterogeneity during disease [105]. To date, several dozen molecular biomarkers have been proposed and/or validated through various techniques, and have recently been reviewed [106,107]. Multiplexing protein detection methods, like antibody array platforms, are expected to be a great tool for future MS diagnosis and prognosis, especially given the current lack of knowledge on disease generation, the heterogeneity during disease, and the need for detection methods of drug treatment efficacies.

Inflammatory bowel disease (IBD) involves chronic inflammation of the gastrointestinal (GI) tract, which affects more than 1.4 million people in United States alone. IBD primarily includes two main distinguishable entities, ulcerative colitis (UC) and CD. While the etiology of IBD remains unknown, the diseases are considered autoimmune in nature, as the immune system attacks elements of the digestive system. Biomarkers in current clinical application include inflammatory markers (CRP), antibodies targeting microbial antigens, autoantibodies (ASCA, pANCA), and fecal proteins (e.g., calprotectin and lactoferrin) [108,109]. However, they are far from ideal in specificity and sensitivity. Antibody arrays were first applied by

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Kader *et al.*, for the study of 65 CD and 23 UC patients, and this group identified four cytokines (placental growth factor (PLGF), IL-7, IL-12p40, and TGF-β1) that were significantly elevated in patients with clinical remission compared to those experiencing active disease [110]. With murine models, Alex *et al.* compared the TH1, TH2, and TH17 cytokine profiles in chemical DSS (UC-like) and TNBS (CD-like) induced murine colitis in IBD. Their results showed that different diseases have distinct cytokine profiles: TNBS colitis had increased TH1 and TH17 responses, while the DSS colitis had predominantly a TH2-mediated inflammatory response [111]. Moreover, a 5-cytokine panel (IL-4, IL-6, IL-12, IL-17, and IFN-γ) can be sufficiently used to distinguish unaffected controls from diseased, as well as to distinguish one disease type from another. Through analysis of cytokine profiles in UC and CD serum samples, Knutson *et al.* found that UC patients showed strong neutrophil activity, while CD had both macrophage and neutrophil activity, indicating the importance and potential role of innate immunity in human IBD [112]. Interestingly, distinct cytokine profiles were also reported for plasmacytoid dendritic cells in the mesenteric lymph nodes of IBD patients, suggesting that eventual disease outcomes might originate in the dendritic cell compartment [113]. Screening a panel of 42 analytes in 38 healthy controls compared against 137 UC patient serum and tissue samples, Coburn *et al.* found that Eotaxin-1 was increased in both sample types in patients with active UC compared to the healthy controls [114]. In a similar research cohort of 67 UC patients together with 21 healthy controls, higher IL-8 was also reported in UC patients, and its expression level was significantly correlated with disease activity [115]. Finally, serum IL-17A level was also reported to correlate with clinical disease severity, and its expression could predict the course of disease, together implying the variety of increased signals that together can affect UC disease, and the inherent need for a multiplexed methodology [116].

Cytokine antibody arrays have been reported in many publications as a useful tool to study the complexity of immunodeficiency diseases. Human immunodeficiency virus (HIV) is a life-long chronic disease that primarily targets the CD4 T cell compartment during infection. This targeting slowly culls off the gut CD4 T cell compartment, and eventually the CD4 T cell compartment entirely, leaving the host unable to fight off the most basic of infections. Through analyzing the profile of 120 cytokines and chemokines in human CSF, Meeker *et al.* showed that increased inflammatory cytokines and chemokines in HIV-infected patients correlated poorly with neurological status, possibly suggesting a marker for differential

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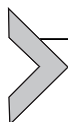
treatment [117]. However, the severity of neurological disease correlated very well with the decline in growth factors, especially neurotrophin-3 (NT-3), indicating the loss of neuroprotection in HIV-infected patients. Given HIV's interaction with multiple cells types (dendritic cells, macrophages, and T cells), understanding how the virus alters their cytokine profiles, and any subsequent disease state changes that occur, will be important to monitor in future studies.



## 8. ANTIBODY ARRAY IN CANCER DISCOVERY

In 2006, lung, breast, and prostate cancer were listed as the leading causes of cancer deaths in the United States and developing countries [118]. Cancer remains a serious threat to public health, responsible for around 25% of deaths annually. As a disease, cancer is driven by both genetic and epigenetic factors, and manifests on a cellular level as a cell or population resisting apoptotic signals, increased angiogenesis, cellular invasion, and eventual metastasis. These are caused, in large part, by altered signaling pathways, which are responsible for the behavior and progression of the disease, including development, progression, metastatic behavior, and recurrence. Many biological functions impact the tumor environment in a localized manner, but the resultant perturbations and inflammation often lead to more global changes in the sera and plasma protein profiles. Identifying cancer-associated protein profile changes, particularly between cancer and noncancerous patients, would provide valuable information for the detection and treatment of this disease, especially if such biomarkers could be identified in patient sera. In order to better identify types of cancer or the stages of the disease in an effort at improving patient treatment, the goal of modern cancer proteomics is to identify specific biomarkers for cancer.

Empirical observations were the basis of early discoveries of cancer biomarkers. The first report of a cancer marker was made in 1848, with 75% of patients from a myeloma study displaying the light chain of immunoglobulin. In the mid-1900s, researchers were able to identify many hormones, enzymes, and other proteins that serve as biomarkers for various cancers due to their significant alterations in the diseased states. The discovery of CEA and alpha-fetoprotein brought about the modern age of monitoring malignant disease in the 1960s, and shortly after lead to the discovery of prostate-specific antigen (PSA) in the 1980s [52]. Hybridoma technology again altered the scope and capabilities of cancer monitoring, during the 1980s, with the development of the ovarian epithelial cancer marker

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carbohydrate antigen 125 (CA-125), and the ability to generate high yield clonal antibodies with known specificity. Presently, more than 20 tumor markers have been fully characterized and are in clinical use, with plenty more to come in the coming age of biomarker discovery. Given cancer's multifactorial disease development, technologies that are able to simultaneously study multiple cytokines will be the lead tool in novel biomarker identification.

Recently, planar antibody arrays have been utilized in a wide range of cancer studies. These include efforts to search for novel cancer-specific markers for diagnosis and prognosis, efficacy studies to search for antitumor agents, elucidating pathways and mechanisms of cancer development and progression, and in preclinical studies with small sample sizes [119,120]. Ovarian cancer is the third most common cancer and a leading cause of death among women in the United States and Europe, and has a high mortality rate due to the oft late-stage diagnosis [121]. Currently, CA-125 and imaging are the main and often sole approaches used during ovarian cancer screening. A recent study comparing the serum samples from ovarian cancer patients, benign ovarian mass patients, and healthy controls were screened with a direct-labeling antibody array interrogating 507 different human proteins. This study identified a small 6-marker cytokine target panel that could significantly distinguish ovarian cancer patients from normal healthy controls in patient serum [122]. Interestingly, these six targets, betacellulin (BTC), endothelin, IL-2 receptor alpha, osteoactivin, osteoprotegerin, and VEGFD, are all involved with angiogenesis, suggesting a potentially early diagnostic marker group.

Breast cancer is the most common and second deadliest cancer in females in the United States [123]. Because of its heterogeneity, more attention is being given to the search for multiple biomarker profiles that would allow for more accurate diagnosis, treatment monitoring and, hopefully, lead to improved prognoses in patients. Using antibody array technology, several studies have identified unique protein expression profiles in breast cancer cell lines [124]. A study using an antibody-based array measured the levels of 35 proteins in a large cohort of 98 breast cancer patients and 96 cancer-free subjects, identified discriminating markers for a population of cancer/cancer-free subjects [125]. Markers for soluble CD40 ligand, EGF, and proapoliprotein A1 were increased in cancer patients, while soluble VCAM-1, vitamin D binding protein, and vitronectin were significantly decreased in cancer patients. This initial panel was also then used to independently probe a separate patient sample group, and with multivariate

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analysis had a sensitivity for detection of 87–91%. Most interesting is this combination of cytokine markers detected early stage breast cancer with surprising sensitivity. This study highlights the viability of antibody array technology for the early detection of breast cancer, in conjunction or separate from traditional evaluation methods, as well as identifying expression profiles that could prove informative as drug targets or prognostic indicators in combating breast cancer.

Bladder cancer is another common malignancy with poor clinical prognosis once the cancer invades the surrounding tissues. By using a protein profiling technique, a 254-target cytokine antibody array was designed to screen a sample population of bladder cancer and healthy controls [126]. The array correctly classified 93% of cancer patients in the first sample group, and this array was then narrowed down to a smaller second antibody array of 144 cytokine targets. This antibody array could predictably project how a bladder cancer patients serum profile related to the patients overall survival, with c-Met as the top predictor of cancer as well as cancer grade, stage, and survival. Validation analyses with ELISA and immunohistochemistry on tissue microarrays confirmed the relevance of these identified proteins for tumor progression, confirming the potential for noninvasive antibody arrays to be used as predictive biomarkers of disease and clinical outcome.

The tyrosine kinase c-Met is the protein product of the c-Met proto-oncogene, and is a hepatocyte growth factor receptor (HGF receptor). It has been associated with a variety of cancer gene products and regulatory proteins, and scientists believe that c-Met is closely related to the occurrence and metastasis of cancer. Studies have found that many cancer patients have elevated levels of c-Met, or increased gene amplification during the course of tumor development and metastasis. Under physiological conditions, the c-Met receptor and HGF binding rapidly initiates physiological effects on the signaled cells [127]. Meanwhile, tumor surface expression of c-Met and HGF, or localized expression of either, leads to the formation of a positive feedback, further allowing tumor growth and invasion of nearby tissues [128]. This positive feedback system has been confirmed in tumors of the nervous system, osteosarcoma, glioblastoma, and other malignancies [129]. Additionally, You and Rountree investigated the significance of c-Met as a biomarker for metastatic and stemness properties in hepatocellular carcinoma (HCC) patients [130]. The identified that c-Met inhibitors induced a substantial level of apoptosis *in vitro*, a finding linked in part to decreased phosphorylation of Akt and Erk. Globally, these changes also resulted in increased epithelial phenotypes via increased E-Cadherin

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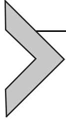
expression. Together, this indicates that c-Met may serve as both a potential biomarker in HCC, and that the HGF/c-Met pathway should be explored as a personalized treatment of patients with HCC or other related diseases where c-Met may be involved. This strategy provides experimental evidence for the use of several integrated technologies, together functioning to strengthen the process of biomarker discovery.



## 9. KIDNEY DISEASES

The primary purpose of the kidney is to filter your blood, thereby removing wastes and excess water to make urine. However, other primary functions include the normalization of blood pressure and the production of hormones for the body. During kidney disease, however, the kidneys are damaged, thereby limiting the removal of excess water and waste, decreasing the ability to maintain normal blood pressures and also altering homeostatic hormonal levels throughout the body. Kidney damage can occur as either AKI or chronic kidney disease (CKD). AKI refers to a sudden decrease in kidney function as measured by changes in serum creatinine concentration and/or urine output. The main cause is the acute apoptosis of renal tubular cells, and is associated with a high risk (>30%) of short-term mortality in severe cases. CKD is the progressive loss in renal function over a period of months or years, following AKI disease progression.

The three most common causes of CKD are diabetes mellitus, hypertension, and glomerulonephritis. Both AKI and CKD may lead to kidney failure, ultimately requiring kidney transplantation. Kidney transplantation is an increasingly successful surgical intervention for ESRD. The RIFLE (risk, injury, failure, loss, and ESRD) criteria, based on serum creatinine levels, are a useful way of assessing successive stages of kidney disease development, but creatinine (Cr) measurements are insufficiently sensitive for detecting early intrarenal lesions. Serum Cr levels are also influenced by many nonrenal factors, limiting the field of vision this marker has with regards to kidney diseases. Furthermore, the serum Cr level does not increase in the early stages of injury, and is therefore, unable to reflect early or minor injuries, which may lead to chronic irreversible damage. Renal biopsy is the sole tool for clinical detection of renal transplantation rejection, and given the sheer invasiveness of this procedure, the problem facing clinicians is how to know when a needle biopsy is warranted.

Efforts have been made to discover some promising protein biomarkers to add significant diagnostic and clinical value to the conventional blood

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creatinine test, especially when these samples types come from noninvasive sources like serum and urine. Recently, seven urinary proteins (albumin, β2-microglobulin, clusterin, kidney injury molecule-1, trefoil factor, total urinary protein, and serum cystatin C) were approved by FDA and European Medicines Agency as markers to support the safety assessment of acute renal tubule and glomerular injury in rat toxicology studies [131]. NGAL, KIM-1, and cystatin C have been largely considered as potential biomarkers for the early monitoring of kidney injury in human [132,133]. Those biomarkers were individually assessed or validated by single-analyte detection methods, like ELISA, Western blot, etc. However, no individual biomarker has good enough performance to solely monitor early kidney injury. Multianalyte technology can overcome the shortcomings from single-analyte detection method, and increase the breadth of potential biomarkers that can be simultaneously evaluated, especially when multiple prognostic factors can be combined together in a simultaneous screening test.

To diagnose and monitor acute kidney rejection, Hu *et al.* used a large human cytokine antibody array to profile 120 urine proteins from renal transplants experiencing acute rejection (AR). The results showed the transplanted kidneys with AR produce cytokines and chemokines that are either absent or deficient in normal healthy individuals. Twenty-three target proteins were selected for further analysis by multiplex assay, where 11 proteins were significantly elevated in AR, but not in healthy individuals. Combinatorial analysis of urinary IP-10, MIG, MIP-1δ, and OPG can differentiate acute renal injury (AR, acute tubular necrosis, and BK viral nephropathy), borderline rejection, and chronic allograft nephropathy from stable graft function and normal renal function [134]. Srivastava *et al.* used a 507 target antibody array to profile urinary proteins from renal transplants and healthy individuals. Twelve urine proteins were significantly increased (≥twofold change) in the acute and chronic rejection groups when compared to healthy control and stable renal transplant groups. Meanwhile 12 proteins were significantly decreased in acute and chronic rejection groups. Reverse capture protein microarrays further confirmed elevated expression of ANXA11, integrins β3 and α3, and TNF-α in kidney allograft rejection cases, suggesting that these four proteins could be candidate biomarkers for predicting renal rejection [135].

There is a need for reliable and sensitive biomarkers for renal impairments to detect early signs of kidney toxicity and to monitor progression of disease in order to best moderate any potential negative patient outcome. Neiman *et al.* used an antibody array to profile plasma samples from patients

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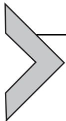
with four types of kidney disorders: glomerulonephritis, diabetic nephropathy, obstructive uropathy, and analgesic abuse. Human fibulin-1 showed significantly increased levels in the glomerulonephritis patient group compared to the controls and other renal disorders. A verification study confirmed fibulin-1 elevation in glomerulonephritis patients, suggesting plasma fibulin-1 as a viable biomarker to monitor kidney malfunction or kidney injury in this setting [136]. Liu *et al.* used a fully quantitative antibody array to investigate the association between urine cytokine levels as it relates to CKD. The results identified that MCP-1, RANTES, TIMP-1, TNF-α, VEGF, E-selection, Fas, ICAM-1, IL-2, MMP2, and TGF-β all were significantly increased in CKD, compared to normal controls. The correlation between the quantified antibody array platform and single-target ELISAs were 0.976 ($p < 0.001$) and 0.939 ($p < 0.001$) for MCP-1 and TNF-α, respectively, confirming the performance validity of antibody arrays for multicytokine detection. These newer antibody arrays allow a fully quantitative measurement of precious samples that go far beyond the mere correlative fold increase changes provided in previous arrays, providing yet another facet to consider with to simultaneous multicytokine detection [137].



## 10. OTHER DISEASES WHERE ANTIBODY ARRAYS HAVE CONTRIBUTED TO BIOMARKER IDENTIFICATION

### 10.1 Ocular disease

Dry eye disease (DED) is a chronic, multifactorial disorder of the ocular surface that affects up to 100 million people worldwide [138]. Although DED can be diagnosed through functional tests, they all have limitations with respect to their selectivity and specificity, and overall they give little indication of a patients' disease severity as they tend to reflect only late changes induced by the disease. For these reasons, the identification of dry eye biomarkers could be potentially useful in clinical practice for diagnosis, prognosis, and monitoring the effectiveness of treatment of DED patients. It has recently been postulated, and subsequently shown experimentally, that inflammation plays a prominent role in the pathogenesis disease, or at least a portion of disease etiology [139]. Numerous groups have used antibody arrays to study the cytokine profiles in tear samples. In fact, many inflammatory cytokines including IL-1, -4, -5, -6, -8, -10, -12, -13, -17, TNF-α, and IFN-γ, were recently reported to have increased expression in DED patients, confirming a role for inflammation in either DED or as a marker

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for disease [140]. Massingale *et al.* found that disease severity was significantly associated with the overall cytokine expression level [141]. VanDerMeid *et al.* reported that the tear inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNF-α) and MMP (-1, -2, -7, -9, and -10) correlated well with the standard Schirmer Strip measurement and tear osmolarity, but were not very reflective of results with TBUT and OSDI tests [142].

Related to the Schirmer Strip test finding, Lee *et al.* found that the cytokine expression profiles in the tears of in Sjogren syndrome DED is different from non-Sjogren syndrome DED [143]. Sjogren's syndrome is classified by a Schirmer Strip test score of below 4 mm of tear production, and is related to an autoimmune disease targeting eye exocrine glands. This group also correlated IL-17 levels with the Schirmer test and TBUT test, suggesting a potential disease biomarker. More recently, Benito *et al.* compared the expression level of 18 cytokines in different tear samples from different time points and different days, to see if there was significant variation from patient sample to patient sample. They found that tear cytokines can be measured reproducibly over time with most not having significant inter- and intraday variability [144]. A 4-cytokine array (IL-1β, IL-6, IFN-γ, and TNF-α) was recently validated as a noninvasive biomarker for ocular surface diseases, suggesting yet another use of antibody arrays for various eye diseases [145].

## 10.2 Oral disease

Periodontal disease (PDD) is a gum disease which derives from a chronic bacterial infection within the oral cavity. These symptoms range from simple gum inflammation (gingivitis) to periodontitis which results in major damage to the soft tissue and bone underlying and surrounding the teeth. In the worst cases, teeth are eventually lost due to the severity of the damage. Because of the irreversible nature of periodontitis, early diagnosis, and treatment is critical for the long-term health of the patient. Traditional clinical measurements include probing pocket depth, bleeding on probing, clinical attachment loss, plaque index, and radiographs among others. While such methods are useful for the staging of PDD, they are only indicators of previous disease status rather than the present disease activity. As such, there is a need for the development of new diagnostic tests that can reflect the status of an active disease state, which is useful for disease diagnosis, prognosis, and monitoring the effectiveness of any periodontal therapy.

Due to its simple and noninvasive nature, saliva has been increasingly evaluated as a diagnostic fluid for detecting multiple diseases including

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PDD [146]. Along this vein, salivary levels of IL-1β, MMP-8, OPG, and MIP-1α were measured and these were identified to strongly reflect the disease severity of chronic periodontitis, as well as reflect the outcome of oral hygiene therapy [147]. On the other hand, the results can vary since many factors will affect cytokine levels within the saliva such as time, state of salivary gland simulation, interference from dietary constituents, and overall oral health status. These are factors that must be considered during collection and subsequent profiling of saliva cytokine levels.

Salivary cytokine profile is also likely to be a more relevant sample type for PDD, as it has been reported to be different from plasma [148] and serum [149]. Gingival crevicular fluid (GCF) has also become a very relevant and widely used sample type for PDD research. GCF is a bodily fluid transuded from periodontal tissues into the gingival crevice and periodontal pocket in very minute volumes. The constituents of GCF originate from serum, gingival tissues, and from both bacterial and host response cells, together reflecting the local biology and physiology of the tissues. Of note is that GCF can be easily collected by noninvasive means, via insertion of absorbent paper strips or via micropipettes. As a result, proteins in GCF have been the ideal and hot targets pursued for candidate disease-specific biomarker research for the last several decades.

Most analyzed PDD-related proteins in GCF are inflammatory cytokines (e.g., IL-1β, -6, -8, -10, -12, IFN-γ, TNF-α, and CRP); MMPs (e.g., -8, -9, and -13) and their inhibitors (TIMPs); bone metabolism related cytokines (e.g., OPG, OPN, RANK, and RANKL); and other enzymes (e.g., alkaline phosphatase and aspartate aminotransferase) [150]. Fiorini compared the cytokine expression level of six cytokines (IL-1β, -6, -8, -10, -12, TNF-α) in GCF and serum. With the exception of all the other cytokines were found to be significantly higher in GCF relative to serum [151]. GCF levels of IFN-γ, IL-17 were found to be significantly increased in inflamed sites in patients with PDD [152,153], and following periodontal therapy a separate group reported that a number of proinflammatory cytokines in GCF were considerably reduced [154]. Reis *et al.* also confirmed that the IL-1α, IL-1β, and IL-6 levels were significantly reduced after nonsurgical periodontal therapy, supporting these markers as a diagnostic tool [155]. Using a large 40-cytokine panel, Shimada *et al.* studied GCF samples from PDD patients in a periodontal therapy program, and compared their expression levels with other diagnostic methods (PDs, BOP, and the presence of pathogen). Nine markers in diseased sites, MMP-3, IL-1β, IL-21, RANTES, IP-10, VCAM-1, PLGF, VEGFA, and TRAIL, were significantly higher than in healthy sites,

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suggesting even local cytokine levels are disparate across the oral surface. Notably, IL-1β levels in GCF correlates well with all the other detection parameters, and might be suitable solely as a diagnostic measure [156].

## 10.3 Orphan disease

Orphan disease is the nomenclature used to describe a variety of rare disease that affect about 1 in every 1500 people in the United States. There are more than 7000 recognized orphan diseases which affect some 25 million Americans or 350 million people worldwide at any given time. Thomson Reuters reports that the compounded annual growth rate for orphan disease therapeutics is much higher than that of nonorphan drugs (26% vs. 20%), and accounts for 22% of current drug sales, with a current global value of $50 billion. Due to the lucrative market, more efforts have been done in recent year in the search of suitable biomarkers for disease diagnosis, prognosis, and therapeutically targets.

Muscular dystrophies (MD) are a group of more than 30 genetic orphan diseases characterized by progressively deteriorating weakness and degeneration of the skeletal muscles that control movement. The immune system plays a pivotal role in the pathogenesis of MD, and is highlighted in a study by Baird *et al.* using a multiplex cytokine assay to identify three significantly elevated cytokines (MCP-1, IL-1ra, and ICAM-1) during MD disease [157,158]. Many chemokines were also upregulated in dystrophic muscle [159]. In another recent publication, the MD serological biomarkers were thoroughly analyzed through the antibody-based array platform measuring the serum or plasma levels of 315 target proteins [160]. Measuring four different diagnostic categories (Duchenne MD, Becker MD, healthy control, and asymptomatic female carriers) their results showed that a group of muscle-specific proteins were highly expressed in MD patients. Moreover, the results demonstrated that serological biomarkers can be efficiently applied to separate each diagnostic category. A panel of four proteins (CA3, MYL3, MDH2, and ETFA) can separate Duchenne MD patients from healthy controls and female carriers, whereas CA3 allowed for separation between Duchenne MD and Becker MD patients. Two proteins (MDH2 and MYL3) could separate BMD patients and healthy controls. Together this suggests these serum biomarkers alone can independently distinguish between the various MD disease and their healthy control counterparts and might afford new diagnostic tests of therapeutic targets. Additionally, the same group identified a 9-protein profile that correlates

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with disease progression and severity in age-matched subcohorts, helping to identify early the patients with the most needs [160].

## 10.4 Infectious disease

Sepsis is the most important cause of morbidity and mortality in the intensive care unit, resulting from a variety of infectious agents (bacterial, viral, fungal, or parasitic) ultimately leading to a pathogenic inflammatory response. The septic response is an extremely complex chain of events involving inflammatory and antiinflammatory processes, all due to the infectious agent's travel from the initial site infection to other organs via the bloodstream. Severe septic shock can give rise to multiple organ dysfunction syndrome and eventual patient death. While anyone can develop sepsis, it is most common in infants, children, the elderly, and people with weakened immune systems. The diagnosis of sepsis and evaluation of its severity is complicated due to its general nonspecific nature and the variety of signs and symptoms. Biomarkers of sepsis which can reflect the severity of sepsis, or differentiate between bacterial from viral from fungal infections, or differentiate between systemic sepsis and local infection, are all undoubtedly useful for disease diagnosis, prognosis, and monitoring the effectiveness of antibiotic therapies.

Clinically, CRP and procalcitonin (PCT) have been routinely used for monitoring potentially septic conditions. However, there are limitations to the use of just CRP and PCT for assessing the severity and predicting prognosis in septic patients, which prompts a need for further identification of better biomarkers of septic situations. Most analyzed sepsis biomarkers include inflammatory factors, cell markers, receptors, coagulation markers, and biomarkers for vascular endothelial damage and organ dysfunction [161]. Recently, Holub *et al.* reported that serum IL-1ra level correlates well with CRP and PCT, and it can be joined from other cytokines (IL-2, -6, -8, TNF-α) to differentiate bacterial infection from viral infection [162]. Plasma inflammatory cytokines were first reported to be higher in sepsis patients than healthy controls [163]. Later, Bozza *et al.* analyzed 17 cytokines from 60 patients' plasma with different septic disease severity, and identified panels of cytokines that can differentiate septic shock patients with severe sepsis patients [164]. Additionally, their concentrations are associated with severity and potential development of organ dysfunction. A multicenter effort of screening nine biomarkers from a large cohort of 971 patients with suspected sepsis revealed that a simple three-marker panel (NGAL, protein C, and IL-1ra) can be used to predict severe sepsis, septic shock, and death with considerable accuracy (0.80, 0.77, and 0.79, respectively) [165].

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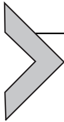
Despite these successes, Lvovschi *et al.* studied a 25-cytokine marker panel in 126 patients, and found that no cytokine profiles can be used to differentiate SIRS, severe sepsis, and septic shock in this cohort group [166]. These disparate results could have arisen from the different sample source used in these studies: plasma for the Bozza groups and serum for the Lvovschi group, but further research is needed to clarify this discrepancy. Supporting such a potential hypothesis on the differential results, Wong *et al.* reported that serum and plasma had different cytokine profiles and their data are not interchangeable [167].



## 11. OTHER ARRAY TECHNOLOGIES

### 11.1 Glycan arrays

Glycan-binding proteins (GBPs) play a critical role in many biological processes, including cell communication, immune recognition, cancer development, and microorganismal infections. However, GBPs remain a largely untapped mechanism for biomarker detection and discovery. Glycan arrays, which contain numerous carbohydrates or carbohydrate conjugates on a solid support, have become a powerful high-throughput tool for rapidly discovering new biomarkers, studying the interactions of carbohydrates with a variety of macromolecules, and providing valuable insights into the biological roles of carbohydrates. As we learn more and more about the proteome and glycome of various disease states, these arrays have the potential to discover the next line of disease biomarkers that detect not only the alterations in protein levels, but also the inherent posttranslational modifications that occur on these proteins.

A number of groups have constructed and used glycan arrays to characterize the specificity of GBPs and to identify diagnostic and prognostic biomarkers in human diseases including cancer, infectious diseases, and autoimmune diseases [168,169]. For example, using an *O*-glycopeptide microarray, Wandall *et al.* demonstrated the presence of higher levels of antibodies to aberrantly *O*-glycosylated Mucin 1 (MUC1) in patients with breast, ovarian, and prostate cancer compared to that of healthy controls [170]. Moreover, this group has also constructed an expanded glycopeptide array displaying a comprehensive library of glycopeptides and glycoproteins derived from a panel of human mucins, and used this array to profile antibodies in serum of cancer patients [171]. They noted that several cancer-associated IgA and IgG antibodies in patients with CRC compared to healthy controls that were targeted to aberrant glycopeptides of MUC1 and Mucin 4. They detected these cancer-associated antibodies in 79% of

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CRC patients with a specificity of 92%, marking these associated aberrations with a fruitful source of sensitive potential biomarkers for the early detection of cancer. F77 antigen is a novel, biologically important, and clinically relevant prostate cancer-associated molecule. However, its direct carbohydrate sequence was elusive. Recently, Gao *et al.* have determined the carbohydrate sequence of F77 antigen using a glycan array containing 492 sequence-defined lipid-linked glycan probes: glycolipids and neoglycolipids [172]. Combined with the *O*-glycome designer arrays and MS approaches, Gao *et al.* have revealed that F77 antigen is expressed on blood group H on a 6-linked branch of a poly-*N*-acetyllactosamine backbone. Their results strongly support the application of F77 antigen as a biomarker to detect F77-positive circulating prostate cancer-derived glycoproteins and tumor cells, as well as how glycan array technology can be used to screen for carbohydrate moiety changes on important biomarkers.

Over the last few years, there have been numerous applications of glycan arrays and have already produced many new biomarker candidates for a wide range of applications, most notably in the field of cancer. With new techniques coming to fruition for glycan detection, like the ability to detect specific glycosylations on a pool of protein samples, new biomarkers are on the horizon. However, current glycan arrays can detect only a small fraction of the glycans found in nature; therefore, new methods for the synthesis, isolation, and characterization of glycans are required to expand the glycan libraries.

## 11.2 Protein/peptide array

A protein/peptide array is a high-throughput method used to study binding properties, functionality, and kinetics of protein–protein or protein–compound interactions [173]. Protein/peptide arrays immobilize individual purified recombinant proteins on the microarray surface, and then are probed by samples of small compounds, proteins, and/or antibodies. They are often used to search for targets of an enzyme (like kinase, phosphatase, protease, acetyltransferase, histone deacetylase, etc.), to search for binding partner of a protein, to map an antibody epitope, to find key residues for protein binding, or to identify the presence of autoantibodies.

Price *et al.* examined SLE patient serum using a nitrocellulose-surface microarray containing human cytokines, chemokines, and other circulating proteins [174]. They found that elevated IgG autoantibody reactivity to B cell-activating factor (BAFF) was associated with SLE compared with

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control samples. Additionally, they noted that BAFF reactivity correlated with the severity of disease-associated features, marking BAFF as a potential SLE biomarker. More recently, Dai's laboratory has developed a new peptide microarray platform constructed on nanostructured plasmonic gold films [175]. This gold platform utilizes spontaneously adsorbed avidin for immobilization of biotin-conjugated peptides and biotinylated branched polyethylene glycol stars to minimize nonspecific binding background signal. Dai and colleagues then profiled human antibodies in the sera of SLE patients using an integrated histone peptide and whole antigen array, and showed accurate profile differentiation of SLE patients from healthy individuals. This new array technology significantly enhances the sensitivity of protein/peptide array, and could be extended into the immunoassay fields. While protein/peptide array has great applications on basic, clinical, and pharmaceutical research, and does not require the identification of specific antibodies, the need for protein purification and peptide synthesis are technically challenging and time consuming.

## 11.3 Nucleic acid programmable protein arrays

To overcome the many obstacles and challenges faced by traditional methods of protein array production, such as protein purification and stability, La-Baer and colleagues have developed the Nucleic Acid Programmable Protein Arrays (NAPPA) by printing complementary DNAs onto glass slides and then translating target proteins with mammalian reticulocyte lysate [176]. GST tags fused to the proteins allowed them to be immobilized *in situ*. Using high-density NAPPA protein microarrays expressing 4988 candidate tumor antigens, Labaer *et al.* reported a signature of autoantibody biomarkers for detection of early stage inflammatory breast cancer. Twenty-eight antigens were further confirmed as biomarker candidates using the independent serum cohort, confirming the capacity of multiplexed array platforms for the identification of potentially lifesaving cancer biomarkers [176].

Recently, Miersch *et al.* have demonstrated the application of NAPPA to identify novel autoantigens in T1D [177]. In the first stage, the immunoreactivity was compared between T1D cases and healthy controls against about 6000 human proteins. Of the 750 genes showing higher signal intensities in patients, further testing revealed a 26-marker panel of novel autoantigens and a known T1D-associated autoantigen. The presence of autoantibodies to dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) was further validated in this same cohort as a marker for T1D. These

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new biomarkers will help illuminate the pathophysiology of T1D and enhance early diagnosis and provide additional avenues for therapeutic intervention.

## 11.4 A reverse phase protein array

A reverse phase protein array (RPPA) is a protein array designed to measure protein expression and modification levels in a large number of biological samples simultaneously, and in a quantitative manner. Small amounts of cellular lysates or body fluids are immobilized on individual spots on an array that is then incubated with a single-specific antibody to detect expression or modification of the target protein across many samples. Hundreds to thousands of samples can be printed on one microarray. Thus, RPPA is a powerful high-throughput tool for studying expression/modification of important biomarkers in human diseases.

Paweletz and collaborators have first developed an RPPA array of cell lysates from prostate cancer specimens microdissected to represent tissue cell populations [178]. They have analyzed the state of several prosurvival checkpoint proteins, and discovered that prostate cancer progression was associated with increased phosphorylation of AKT, suppression of apoptotic pathways, and decreased phosphorylation of ERK. Using RPPA array, Gujral *et al.* have recently profiled signaling proteins in 56 breast cancers compared to matched normal tissue [179]. Seventy-one of the 100 antibodies yielded signals above background, and 54 out of 71 showed significant variation between tumor and normal tissue, suggesting multiple aberrant cellular changes. The patterns of these changes are consistent with known mechanisms of oncogenesis-related signaling networks. They further explored in detail one tumor-associated pattern that involves changes in the abundance of the Axl RTK and phosphorylation of the c-Met RTK, and revealed cross-talk between Axl and c-Met. These findings have great potential of therapeutic implications, as they mark potential drug targets as well as prognostic markers of disease.

RPPA has various applications such as quantitative analysis of protein expression in cancer cells, body fluids, or tissues for biomarker profiling, cell signaling analysis, and clinical prognosis and diagnosis. However, the signal generated by RPPA could be generated from unspecific primary or secondary antibody binding. Thus, it is essential to identify specific antibodies and any potential cross-reactivity between antibodies, to ensure values are non-specific reactions. Additionally, follow-up is needed to confirm any data from RPPA, via using traditional assays, such as Western blot.

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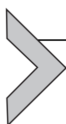
## 11.5 Peptoid arrays

Peptoids, oligomers of *N*-substituted glycines, were developed as peptide mimetics for the generation of chemically diverse libraries of novel molecules, especially some molecules with relatively poor stability. The chemical structure of a peptoid shifts the side chain of peptides from the α-carbon to amide nitrogen group. Due to this unique chemical structure, peptoids have several advantages over peptides: larger selection of side chains; better solubility and cell membrane permeability; excellent resistance to proteolytic cleavage [180]. Peptoid arrays are similar to peptide arrays, as both can be used for detection of novel binding ligands to proteins and antibodies.

Dr. Thomas Kodadek from the Scripps Research Institute identified three novel octameric peptoids which showed strong affinity to IgG from patient serum of ADs than in normal control serum using a 15K peptoid molecules array [181]. They developed and demonstrated a screening technology which can be used to detect serum biomarkers via an array using 10K peptoid molecules on glass slides. Dr. Holger Wenschuh group introduced a method of synthesizing peptoid arrays on cellulose membranes using SPOT techniques [182]. They prepared 8K peptomers peptoids on the membrane array and identified 2 μM ligands for the mAb Tab-2 using this array. Each of these techniques could be explored for discovery of new drug targets for disease, or for biomarker discovery in identifying novel traits inherent to diseases or cancer.



# 12. SUMMARY

The early uses of antibody arrays centered on an inherent ability to prescreen large panels of cytokines in a multiplex platform to identify potential candidates of interest. More recent technology advances now allow for fully quantitative antibody arrays which are currently being used to validate previous findings of biomarker panels, as well as to characterize the physiological levels of these biomarkers in their respective samples. At current pace, clinical usage for diagnostic purposes is on the horizon, and with further antibody pair identification to expand the current selection of detectable biomarkers, as well as technological advances that will improve the accuracy and efficiency of antibody arrays, clinical diagnostic usage could soon become routine.

Overall, the ability of antibody arrays to simultaneously detect multiple proteins, do so in a semi-quantitative or fully quantitative fashion, and with incredibly high sensitivity, specificity, and cost effectiveness have bolstered

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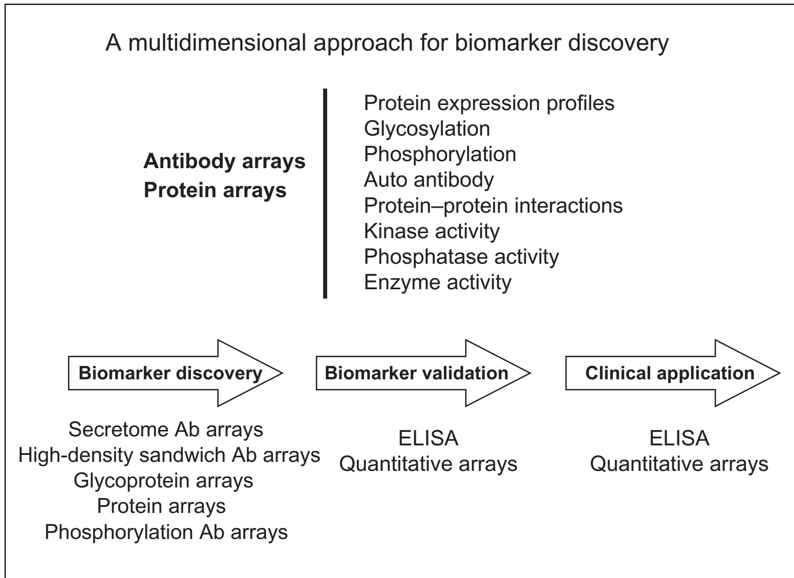


**Figure 6** General outline of antibody arrays methodology.

its standing in the research community. Further, antibody array use in the discovery of numerous cancer and autoimmune biomarkers has validated its use in the field, and marked it as a major tool in biomarker discovery and development. Since its invention in the early 2000s, this ever maturing technology has facilitated thousands of investigators around world in various fields of biomedical research. Through more than a decade of use in research and development, this technology has established multiple disease biomarkers, presented researchers with a number of candidate drug targets, and helped to identify and project outcomes of drug treatments. With sure to come technological advancement the field is set to endure, and the ever evolving realm of biomedical research, the high-throughput multiplexed antibody array platform is set to be at the forefront of biomarker discovery (Fig. 6).

## ACKNOWLEDGMENTS

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