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Review – Part of the Special Issue – Pharmacology in 21st Century Biomedical Research

Biomarkers in pharmacology and drug discovery

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ABSTRACT

Biomarkers, quantitatively measurable indicators of biological or pathogenic processes, once validated play a critical role in disease diagnostics, the prediction of disease progression, and/or monitoring of the response to treatment. They may also represent drug targets. A number of different methods can be used for biomarker discovery and validation, including proteomics methods, metabolomics, imaging, and genome wide association studies (GWASs) and can be analysed using receiver operating characteristic (ROC) plots. The relative utility of single biomarkers compared to biomarker panels is discussed, along with paradigms for biomarker development, the latter in the context of three large-scale biomarker consortia, the Critical Path Predictive Safety Testing Consortium (PSTC), the NCI Early Detection Research Network (EDRN) and the Alzheimer's Disease Neuroimaging Initiative (ADNI). The importance of systematic optimization of many parameters in biomarker analysis, including validation, reproducibility, study design, statistical analysis and avoidance of bias are critical features used by these consortia. Problems including introduction of bias into study designs, data reporting or data analysis are also reviewed.

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1. Introduction

Biomarkers, where these exist and have been appropriately validated [1–7] can play a critical role in biomedical research, drug discovery and development, including:

- Their use as diagnostics to establish the presence of a disease.
- predicting disease progression and the stratification of disease severity.
- assessing and predicting the clinical benefit(s) or toxicity resulting from a therapeutic intervention.
- monitoring treatment response, including target engagement where appropriate.

Biomarkers can be used as part of a personalized medicine paradigm to customize treatment to the specific disease characteristics of an individual patient. They can also be used to better understand disease mechanisms and to identify novel disease targets.

Issues that are addressed in this review include: (i) biomarker discovery, development and analysis that requires a combination of careful study design to avoid distorting or negating results due to bias; (ii) systematic development of assays; (iii) larger scale collaborations involving multiple patient populations to develop and refine robust and reproducible assays and; (iv) for clinical validation [1–7], transparent data analysis (particularly when large datasets are involved), with statistical analyses appropriate for the specific use for which the biomarkers are being developed.

2. Defining biomarkers

Some biomarker definitions are listed in Table 1. These indicate their scope of use, which can include: application to various stages of the drug discovery process; characterization of animal disease models; use in clinical trials to stratify patients; use as diagnostics or companion diagnostics; or as indicators of therapeutic response.

Table 1
Definition of biomarker types.

Term	Definition
Biomarker	objectively measured characteristic evaluated as an indicator of normal biological or pathogenic processes, or pharmacological response(s) to a therapeutic intervention [8]
Surrogate	biomarker intended to substitute for a clinically meaningful endpoint; predicts clinical benefit, harm, or lack of either; a direct measure of how a patient feels, functions or survives [8,9]
Clinical endpoint	a characteristic or variable reflecting how a patient feels, functions, or survives [9]
Diagnostic	biomarker for the existence of (often asymptomatic) disease in an individual
Prognostic	characteristic predicting disease progression or outcome in an untreated individual [10,11]
Predictive	characteristic predicting patient benefit or toxicity from a specific intervention [10]
Pharmacodynamic	biomarker showing direct pharmacological effect of an NCE or drug [10]
Efficacy	characteristic predicting clinical benefit
Toxicity/safety	biomarker predicting clinical risk

Biomarkers, broadly defined, can be a variety of (ideally) quantitatively measured indicators of biological or pathphysiological processes, or the response to therapeutic intervention, including molecular entities, images or other measured activities or properties, or their combination as a biomarker panel. They include proteins, protein modifications, or activities, e.g., enzymes [12]. DNA-based biomarkers include DNA (e.g., circulating DNA can be used to diagnose genetic diseases such as Down's Syndrome in unborn children [13]), single nucleotide polymorphisms (SNPs), gene copy number variations (CNVs; DNA insertions, deletions, rearrangements such as inversions and translocations larger than 1 kb, and insertions/deletions less than 1 kb [14]), mRNA or long non-coding RNA [15]. Epigenetic biomarkers include methylated DNA (e.g., fecal DNA tests for detection of colorectal cancer [16]), microRNA (e.g., a microRNA panel for diagnosis of stage II-IV colon cancer [17]), or modified histones. Other more traditional biomarkers include gross phenotypes such as blood pressure, lung volume, blood sugar and urine volume, cellular metabolites or lipids and other physical measurements including structural (e.g., computed tomography or magnetic resonance) or functional (e.g., positron emission tomography) images as used in neuroimaging [18], or electroencephalograms [19]. Combinations of different biomarker types (e.g., fluid biomarkers, tissue images, allele expression [20], clinical imaging, and molecular biomarkers [21]) may be useful for improved diagnostic accuracy [22,23], particularly in complex disease states [24], and may be more effective than individual biomarkers (see Section 4.1).

The NIH Biomarkers and Surrogate Endpoint Working Group [8] has classified biomarkers into:

- Type 0 (reflects natural history of a disease, correlates with known clinical indices over the full range of disease states),
- Type I (reflects the effects of therapeutic intervention by the mechanism of action of a drug), and
- Type II biomarkers (surrogate endpoints, whose change predicts clinical benefit).

Biomarkers can also be subdivided by their application. A clinical biomarker is a predetermined, validated characteristic or variable reflecting how a patient feels, functions, or survives after treatment [8]. Surrogate markers or endpoints are intended to substitute for a clinical endpoint, reflect clinical benefit or harm, and/or directly measure patient function or survival. Definitions and examples of cardiovascular biomarkers include use of blood pressure- and/or HDL cholesterol-lowering for cardiovascular drugs [25,26] and the use of glycated hemoglobin (HbA1c) as a surrogate endpoint in diabetes [27]. Validation of surrogate endpoints requires extensive data, including large randomized clinical trials, that must demonstrate that the surrogate is prognostic for the true clinical endpoint, and the effect of therapy on the surrogate predicts its effect on the true endpoint [28]; as a consequence, such markers are rare.

Diagnostic markers are biomarkers for the existence of the disease in an individual; such markers may in some cases be used to specify stages of disease, in which case they are also prognostic biomarkers. For diseases for which effective late-stage disease therapy may not exist, e.g., some of the major cancers, these markers are most beneficial when detected early in the disease process.

While such markers are useful if an effective therapy for the disease exists, in diseases with no current effective therapy, e.g., Alzheimer's disease (AD), diagnostic markers may be more useful in the context of basic research on the disease process, or for assessing new chemical entities (NCEs) as potential lead therapeutics. Prognostic biomarkers can also be used to predict disease progression or outcome in untreated individuals [10,11]. Examples in different stages of development include microRNAs for predicting the progression of urothelial carcinoma [29], and commercial multigene diagnostic tests [30], e.g., the Oncotype DX™ test, that examines a tumor biopsy or surgical resection panel of 21 genes to predict the 10-year recurrence of estrogen receptor–positive early-stage breast cancer [31]. Predictive biomarkers are also useful for predicting patient benefit (or toxicity) from a specific therapeutic intervention [10] and include amplification of the *HER2/neu* oncogene in early stage breast cancer [32], advanced stage gastric and gastroesophageal junction cancer [33], and ovarian mucinous carcinomas [34], all predicting benefit from the anti-*HER 2* antibody trastuzumab. Biomarkers are also useful in assessing the effects of NCEs, with pharmacodynamic (PD) biomarkers providing evidence of a pharmacological effect of an NCE, e.g., NCE-induced toxicity, NCE-related changes in patient physiology, etc. [10].

2.1. Drug targets and biomarkers

Biomarkers can also be classified by their relationship to drug targets, the majority of which are proteins. In the case that the biomarker is the putative therapeutic target, biomarker status may be predictive for a putative drug response, and co-development of the biomarker (when available) and a putative therapeutic in clinical trials may in some cases accelerate the drug development process [35]. In this case monitoring target activity (where feasible) can allow quantitation of the extent to which the NCE reaches its intended target [2] that may then allow correlation with overall NCE efficacy in disease models.

Some examples of cancer biomarkers that are also drug targets, or which can create a synthetic lethal drug target (BRCA 1 or 2 mutations), are shown in Table 2 (see [54,57]). In each case a specific receptor, its mutation or exon deletion (e.g., estrogen receptor, epidermal growth factor receptor [EGFR], or retinoic acid receptor (RAR) fusion with the promyelocytic leukemia [PML] gene), oncogenic fusion protein (e.g., BCR-ABL, PML-RAR), or mutations of a defined oncogene (BRCA 1 or 2) can predict response to a therapeutic targeted to that protein, or in the case of BRCA 1 or 2, to a key protein (poly ADP ribose polymerase [PARP]) in a redundant second DNA repair pathway, creating a synthetic lethal target. For many of these cancer biomarkers, companion (to the therapeutic) diagnostics are available to test for the presence of the marker, allowing a targeted therapeutic approach to the particular cancer when the marker is present. Use of the biomarker-based companion diagnostic with the appropriate therapeutic agent can improve the response rate for appropriate patients. For chemotherapy-naïve patients with advanced non–small cell lung cancer (NSCLC) and sensitizing EGFR mutations, treatment with the tyrosine kinase inhibitors, erlotinib or gefitinib was associated with a ~70% response rate [41].

2.2. Polypharmacy

Although primary drug targets may be desirable biomarkers when they exist, cellular proteins that actually bind to a drug may be more numerous (polypharmacy), potentially affecting the association of some drug properties with only the putative primary target. In this case additional experiments may be necessary to assign particular properties to the putative primary target (e.g. targeted knockdowns); without these experiments use of a (putative) primary target as a biomarker of the clinical effects of drug action can be misleading. For some drug-biomarker pairs, binding of drug to additional proteins may not have been examined. The kinase inhibitor, imatinib, the analog nilotinib (which is 20-fold more potent inhibiting the putative major target, the fusion BCR-ABL kinase), and dasatinib, which is 200-fold more potent, are used to treat chronic myeloid leukemia (CML) [58]. In chemical proteomics experiments in K562 and CML primary cells, 11, 14 and 38 major interacting proteins were identified for imatinib, nilotinib and dasatinib respectively [58] with all three binding BCR-ABL, BCR and ABL [59]. In contrast to nilotinib, dasatinib exhibits clinical side effects including cytopenia and pleural effusions [60], suggesting that its much larger target profile of 28 kinases in addition to BCR-ABL and ABL, and major immune system regulators, may be responsible for the side effect profile. For nilotinib, the receptor tyrosine kinase, DDR1 (Discoidin

Table 2
Cancer biomarkers, drug targets, and associated companion diagnostics.

Cancer and biomarker	Target and drug/antibody	Companion diagnostic example
Breast cancer; ERα levels [36]; ERα is overexpressed in ~50% of cases [37]	ERα; antagonist tamoxifen	IHC [38]
NSCLC; EGF receptor, exon 19 deletion [39]; receptor-activating mutations in ~10% of cases [40]	EGF receptor; tyrosine kinase inhibitors erlotinib, gefitinib	Roche cobas EGFR Mutation Test [41]; Therascreen EGFR29 [42]
Colorectal cancer; EGF receptor + activating K-RAS mutation [43] seen in 35–45% of cases [43]	EGF receptor; monoclonals cetuximab, panitumumab NOT indicated	Lung Cancer Mutation Panel [41]
Breast cancer; BRCA1 or 2 inactivating mutations [44] seen in 5–10% of cases in Caucasian women [45]	PARP; synthetic lethal inhibitors olaparib, veliparib	BRAC Analysis [44]
Chronic myeloid leukemia; BCR-ABL fusion protein, kinase is always active [46]; seen in ~100% of cases	BCR-ABL kinase; inhibitors imatinib, nilotinib, dasatinib	RT-PCR; karyotyping [47]
Acute PML; PML-RARα fusion protein [48], seen in ~5–8% of PML cases [49]	PML-RAR; all-trans retinoic acid + AsO3	Quantitative RT-PCR [50]
Breast cancer; HER2/neu proto-oncogene amplification, seen in ~20% of cases [51]	HER2/neu; monoclonal trastuzumab	FISH [52]
Metastatic melanoma; B-RAF V600E activating mutation [53], seen in ~30–50% of cases	B-RAF; monoclonal vemurafenib	Roche cobas 4800 RT-PCR assay [42]
NSCLC; EML4-ALK fusion oncogene [54], seen in ~5% of overall cases [55]	EML4-ALK; kinase inhibitor crizotinib	Abbott Vysis ALK Break Apart FISH; IHC [56]

domain receptor family, member 1) was inhibited by clinical concentrations of the drug and identified and validated as a second major target, which may be associated with side effects [59]. For gefitinib, the first approved kinase inhibitor targeting the EGF receptor in NSCLC, 20 previously unknown kinase targets were discovered using a chemical proteomics approach [61]. Of these, GAK (Cyclin G-Associated Kinase), which functions as a negative regulator of EGFR signaling [62], was inhibited (IC_{50} = 90 nM), suggesting it might be an undesired target [61]. These experiments illustrate the need for caution when examining protein biomarkers of drug action without a further examination of drug-binding proteins using methods such as chemical proteomics.

3. Biomarker discovery

An initial step in any biomarker project involves defining the goals for the project. Before starting biomarker discovery, the intended use of the biomarker(s) should be specified (e.g., early detection of disease, effect of a drug on disease outcome, target identification, etc.), as should the tissue source, how the biomarker will be measured (gene expression microarray, immunoassay, proteins discovered by mass spectrometry), the intended patient and control populations, the clinical setting and clinical endpoint(s) of interest, how the biomarker test will be interpreted, and whether existing evidence supports the relevance and potential use of an animal model instead of patient samples [63]. Concerns may exist, for example, in translating mouse cancer models to humans [64,65], or the translation of mouse models of neuropsychiatric diseases including cognitive endpoints [66–68] to AD patients [6]. Use of conveniently available samples not specifically collected as part of a standard protocol for a defined project (“convenience samples”) can lead to bias and problems with validation by independent groups, especially when the diagnosis is not adequately documented [63,69]. Urine, serum, plasma, saliva or sputum may be more readily obtained than tissues requiring biopsy, unless banked clinical tissue samples are available for a project. Since blood perfuses all tissues it theoretically could be considered a default biomarker source, but disease-proximity of the samples obtained for analysis is an important consideration for enrichment of disease-related molecules. In CNS disease states cerebrospinal fluid (CSF) can be used for this purpose [70], while blood may be useful for cardiac diseases, and blood or urine may be appropriate for metabolic, liver (along with bile), or kidney diseases. Gastric biomarkers can utilize gastric juice [71] and oral cancers, saliva [72]. Locally diseased tissue can utilize interstitial fluid [2], and pulmonary disease can be accessed via saliva, sputum or breath condensate. In cases where tissue biopsies are impractical (e.g., live AD patients), samples collected remote from the primary site of disease can provide successful biomarkers (e.g., 170 blood RNA probes representing 133 genes are reported to be able to distinguish AD patients from non-demented controls with 100% sensitivity and 96% specificity [73]). For sample collection and storage for discovery experiments, standard operating procedures for specimen collection and handling are critical, as small differences in processing or handling can have large effects on the reliability of analytical assays and their reproducibility [74]. Part of a biomarker discovery effort should include the *analytic validation* of biomarker assays, i.e., how robustly the assay detects or quantitates the biomarker of interest under different conditions, as well as verification of abundance changes using a method different from that used for discovery and initial quantitation of changes. As an example, Addona et al. [75] included in their mass spectrometry-based protein biomarker discovery pipeline, verification of abundance changes in peripheral blood by Western blotting and ELISA, verification and relative quantitation by multiple reaction monitoring mass spectrometry, and verification by accurate mass inclusion mass spectrometry.

Rifai et al. [2] point out the utility of “gold standard” tissue samples for protein biomarkers, selected not only to be disease-proximal (close to or in direct contact with diseased tissue) to help overcome the 5–7 orders of magnitude lower abundance of many protein biomarkers compared to the most abundant serum/plasma proteins (or to overcome dilution for other biomarker types), but to provide maximum contrast between the non-diseased control and diseased states. Individual patients can in some cases be used as their own controls, with samples collected before and after a particular therapy, or from contralateral organs if only one is diseased [2]. While this approach may optimize the discovery of biomarkers that differ between diseased and non-diseased tissue as many inter-patient variables are removed, if the disease is a systems phenomenon rather than completely localized to the selected tissue, potential biomarkers may be lost. Since all subjects are diseased, in effect this approach relies on extreme bias in patient selection. If biomarker discovery experiments utilize a limited number of patients that are not representative of the eventual targeted population, translation to the latter (which may include many non-diseased individuals and/or individuals with additional confounding factors, e.g., co-morbidities) must be addressed in later stage studies including large numbers of patients with adequate statistical power to meet the goals of the study.

If available, consideration should be given to use of a positive control (e.g., gold standard therapeutic if examining a new drug candidate) to allow comparison with existing biomarkers, and to determine if, for example, observed abundance differences are meaningful. Another consideration involves discovery of biomarkers for diseases known to be heterogeneous, e.g., breast cancer. In these cases a single biomarker or even panel of biomarkers may not be predictive for all patients. In such cases studies to identify biomarkers may require two-fold or larger sample sizes than for homogeneous diseases, as well as use of different statistical selection methods [76]. For protein biomarker candidates, the number of samples analyzed in a non-targeted discovery approach may be in the range of tens of independent samples; tens of samples are also used to confirm differential abundance by a different method(s), while hundreds of samples might be used to examine candidates in larger population-derived samples; thousands of samples would be analyzed in clinical assay development and validation establishing biomarker sensitivity and specificity [2].

Different methods can be utilized to discover biomarkers. Targeted methods rely on prior information on biomarker candidates to be examined in discovery experiments. Non-targeted methods, which may presuppose the category of a potential biomarker (e.g., protein, metabolite or small chemical, SNP, microRNA, etc.) but not their identity include for example proteomics, metabolomics, a variety of imaging methods, gene-expression profiling, genome-wide association studies (GWASs), whole genome sequencing, and examination of epigenetics-related factors such as DNA methylation or microRNA. Some of these are discussed in detail below.

3.1. Targeted methods

Targeted methods, which are not be covered in detail in this review, include the use of established assays, e.g., ELISA, to scan for changes in analytes based on prior knowledge. A strength of this approach is that discovery can start using a well-established assay. As an example, Villeda et al. [77] utilized an array of 66 cytokine,

chemokine or signaling protein ELISA assays to scan for plasma immune system factor changes in a mouse parabiosis model of brain aging. Increases in 6 factors, including the chemokine CCL11, correlated with reduced neurogenesis and impaired learning and memory. In principle, catalogs of proteotypic peptides (mass spectrometry (MS)-detectable peptides identifying unique proteins) can be used with multiple reaction monitoring-MS for a much larger, but still targeted, screen for biomarker discovery [78]. Protein antigen microarrays (in a sense targeted if only a subset of the proteome is included) can be used to examine potential autoantibody biomarkers, e.g., as in early-stage cancer [79]. Oka et al. [80] utilized a solid phase antibody array to screen 274 proteins for biomarkers of acute onset interstitial lung disease, finding 4 proteins (including matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloprotease-1 (TIMP-1) increased, and 3 proteins decreased in serum of diseased patients.

3.2. Non-targeted methods

3.2.1. Proteomics: 2D LC/MS/MS

2D LC/MS/MS (two-dimensional liquid chromatography tandem mass spectrometry) generally utilizes two serial orthogonal capillary columns to fractionate peptides by HPLC for direct introduction into a mass spectrometer for analysis [81]. The first is often a strong cation exchange column that can be run offline to fractionate larger amounts of proteins before their proteolysis to peptides that can be separated into batches by their isoelectric point. The second column, e.g., a C18 reversed phase column, chromatographs peptides from each fraction from the first column, separating peptides by their hydrophobicity. The combination of columns can fractionate tens of thousands of peptides (when present) in a single experiment, with identification using tandem MS [82] to identify post-translational modifications as biomarker candidates. The relative quantitation for expression differences in biomarker discovery experiments utilizes: (a) multiplexed stable isotope labeling of the peptides (e.g., by amine-reactive reagents such as Tandem Mass Tags or iTRAQ reagents [83] for comparison of up to 6–8 experiments in one 2D run); (b) incorporation of stable isotope labels directly in cell culture [84]; or (c) a variety of non-multiplexed label-free methods [85], including the exponentially multiplied protein abundance index [86] or spectral counting [87]. Online 2D LC/MS/MS can be automated or semi-automated and is the method of choice for simultaneous identification and relative quantitation of protein expression levels, as well as for deep proteome analysis, typically identifying and quantitating up to ~1000 proteins. Addona et al. [75] utilized offline 2D LC/MS/MS of samples taken directly from patient hearts, to identify 121 protein biomarker candidates that were up-regulated 5-fold or more after planned myocardial infarction, including over 100 novel candidates and 6 known markers of myocardial injury, for detection of myocardial infarction. A subset of 83 proteins was subsequently verified in peripheral plasma, and 52 were prioritized for quantitative assay development.

With the development of high resolution- and rapid duty cycle-MS instruments, the number of proteins that can be both identified with high confidence, and quantitated, has increased to ~8000–11000 in single experiments [88–90]. In the case of estrogen receptor negative breast cancer primary cell lines, 8750 identified and 7800 quantitated proteins were identified, with immunohistochemistry verification of a 52-protein signature on tissue microarrays, leading to stage-specific protein signatures and a proposed overall survival prognostic marker panel of three proteins [89]. Analysis of large proteomes should allow discovery of lower level biomarker candidates and significantly increase the depth of analysis.

3.2.2. Proteomics: SELDI-TOF, MALDI protein profiling

A second MS-based method in wide use involves surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS [91] also known as MALDI (matrix-assisted laser desorption/ionization) protein profiling. This approach is higher throughput and more user-friendly than 2D LC/MS/MS, and involves adsorbing or fractionating peptides or proteins directly on derivatized sample plates for analysis. Desorption of peptides from the samples utilizes a laser and sample matrix, and analysis involves a TOF analyzer, but in principle can utilize other mass analyzers as well. Some of the issues with this method include [92]:

(a) peptides or proteins are not identified as part of standard profiling, thus comparisons involve pattern matching sets of peaks, removing a layer of bioinformatics analysis useful for determining potential biological relevance of markers;
(b) profiles feature highly abundant peptides or proteins observed at the expense of low-abundance species, biasing markers to acute phase response and inflammation-associated proteins [93,94], due, in part, to ion suppression [95,96];
(c) the peak content may become unnecessarily complex when abundant proteins undergo unintended in-source decay [97];
(d) reproducibility of results may be a challenge [98–101], including difficulties with pre-analytical sample treatment [102], peak pre-processing [103], peak quantitation and dynamic range [104], and data analysis.

One of the early studies applying SELDI-TOF analysis to the diagnosis of ovarian cancer [105] suffered from difficulties with analytical methodology, bias in sample collection and storage, and bioinformatics analysis [69,106]. A validation study of use of this methodology for prostate cancer by the Early Detection Research Network (EDRN) showed no diagnostic utility [107]. In light of these issues, the use of SELDI-TOF in biomarker discovery studies should be carefully evaluated before studies are undertaken.

Mass cytometry, which allows simultaneous analysis of dozens of different metal isotope reporter-labeled antibodies bound to single cells, using inductively coupled plasma mass spectrometry and a TOF detector, may in the future be useful for biomarker discovery, for example for autoimmune diseases [292].

3.2.3. Metabolomics

Metabolomics-based biomarkers can be accessed using multiple analytical platforms [108], including LC/MS, GC/MS (gas chromatography/MS), ICP/MS (inductively coupled plasma MS) for analysis of metals, CE/MS (capillary electrophoresis/MS [109], and NMR (nuclear magnetic resonance) [110]. Examination of exhaled volatile organic compounds as metabolites and potential biomarkers utilizes GC-TOF/MS and ion mobility MS among other methods [111]. NMR can currently detect low micromolar analyte concentrations (depending on the number of equivalent protons in a resonance, spectral region and magnet field strength), but is approximately 5 or more orders of magnitude less sensitive than MS, which can detect low to sub-femtomole levels using capillary columns. Combined with MS and MS/MS, NMR can be important for metabolite structure determination [112]. NMR can be used in combination with pattern recognition methods for untargeted metabolomics analysis; the main steps in sample preparation, spectral acquisition, data preprocessing and analysis, including application to biomarker discovery using CSF of multiple sclerosis patients, have been reviewed [112]. A variety of early candidate biomarkers, such as elevated lactate and reduced acetate levels, have been identified in small-scale studies and await analysis in larger patient cohorts. Untargeted global metabolite profiling utilizing high resolution mass spectrometry coupled to UHPLC (ultra high performance liquid chromatography) [113], resulted in

the identification of over 3000 plasma molecules, almost half of which resided in a core metabolome (metabolites; environmental, dietary and endobiome-related chemicals) common to seven mammalian species [114]. Identification of unknown metabolites involves: (i) collection of high mass accuracy MS data that can be useful for determining elemental composition; (ii) MS/MS data useful for identification and structure determination; (iii) chromatographic retention times; (iv) database searching, e.g. METLIN (Metabolite and Tandem MS database; metlin.scripps.edu), HMDB (Human Metabolome Database (hmdb.ca), MMCD (Madison Metabolomics Consortium Database; mmcd.nmrfam.wisc.edu) and others; (v) additional data from NMR or deuterium exchange experiments; and (vi) confirmation with commercial or synthetic standards [115]. Small molecules identified from untargeted approaches include not only host organism metabolites, but nutrition-derived molecules, microbiome-related metabolites, and chemicals from environmental exposure, providing a larger systems-based view of metabolomics [116,117].

Metabolomics and small molecule chemical information can be utilized for pathway mapping utilizing databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes; genome.jp/kegg), Metscape (ncibi.org/gateway/metscape.html), MetaCyc (metacyc.org), etc. Identification of low level metabolites or chemicals can be challenging; many detected peaks may not have database matches; and the lack of authentic standards once a peak is tentatively identified can make validation impractical. As an example, plasma levels of the cholesterol metabolite, desmosterol, and the desmosterol/cholesterol ratio, can be used as AD biomarkers with a ROC (receiver operating characteristic, see Section 4) AUC (area under the curve) of 0.80 [118]. Other examples of metabolites as potential biomarkers in different cancers, age-related macular degeneration, atherosclerosis, diabetes and AD have been reviewed [119]. As with other discovery methods, careful attention to sample preparation [120,121] and data analysis [112,122] is critical to success.

3.2.4. Imaging

A variety of non-invasive (e.g., MRI) and relatively non-invasive (e.g., PET scan) imaging methods have been utilized for biomarker discovery, where the image or image-derived information is used as a "biomarker" or part of a biomarker panel, if there are image-based reproducible (ideally quantitative) differences between the patient states being compared. Imaging may be appropriate when tissue or whole-organ changes are associated with disease pathology, for comparing diseased and control organs, different disease stages, or examining changes after therapy. Neuroimaging has been extensively utilized to examine AD patients, e.g., MRI imaging to examine brain area (hippocampus, entorhinal cortex, corpus callosum) losses in volume during disease progression [18]. [^{18}F]-deoxyglucose PET imaging has been used to examine glucose hypometabolism that correlates with severity of dementia [123] and as a biomarker to predict clinical decline in AD patients with mild-to- moderate disease [124]. The [^{11}C]-labeled ligand Pittsburgh compound B (PiB) binds to aggregated, fibrillar β-amyloid deposits with high affinity [125] and can thus image these deposits in the brain. PiB binding was used as a quantitative phenotype in gene-association analysis to examine the effects of SNPs in 15 amyloid pathway-associated candidate genes on amyloid fibril formation [126]. In this study SNPs most highly associated with PiB uptake were from *DHCR24*, which codes for 24-dehydrocholesterol reductase, an enzyme catalyzing several steps in cholesterol biosynthesis, which is down-regulated in AD [127]. An intronic SNP resulting in higher gene expression resulted in lower PiB binding compared to non-carriers, consistent with the involvement of this gene in amyloid fibril formation and, potentially, with a neuroprotective effect [126]. In a multiparameter study of familial AD, PiB PET imaging of β-amyloid fibrils was detectable 15 years before expected AD symptoms [128].

3.2.5. Mass spectrometry imaging

A different type of imaging involves use of a laser and MALDI matrix to desorb/ionize analytes from thin tissue slices [129], tumor biopsy tissue cores [130], or cells grown on glass slides [131]. From a single imaging "pixel," MS spectra can provide information on hundreds of molecules including (in different experiments) peptides [132], proteins [133,134], drugs [135], metabolites [136] and gangliosides [137], while MS/MS spectra from imaging tandem MSs can identify analytes including peptides [138], lipids and metabolites [139]. The images themselves, or components identified from imaging spectra, may serve as biomarkers [140]. The applicability to larger scale biomarker analysis has been enhanced by advances in instrumentation and in the analysis of large numbers of samples [130]. Analyte detection typically utilizes a TOF mass analyzer on a MALDI-TOF instrument, but can be part of a quadrupole/TOF [135], TOF/TOF [140], or ion mobility/TOF [141] instrument. Other useful sources include a DESI (desorption electrospray ionization) source, that utilizes solvent charged droplets and ions to desorb/ionize analytes, and a secondary ion (SIMS) source, which utilizes an ion or cluster beam to desorb/ionize analytes and has high (sub-micron) lateral resolution [142]. An infrared laser for laser ablation electrospray ionization (LAESI) [143] can also be used for imaging [141]. Using tandem MS, images with improved signal/noise can be obtained by utilizing an MS/MS peak from a selected precursor ion [135]. High lateral resolution (~1 μ) MALDI imaging has been combined with UV confocal microscopy [144] and a high resolution atmospheric pressure imaging source on a Fourier transform instrument, allowing observation of volatile compounds with high quality images [145]. MALDI imaging MS has been integrated with MRI for colon cancer diagnosis [146] and can be extended to whole-body (e.g., rat) imaging of vinblastine [141]. Registration of serial 2D tissue section images allows the construction of 3D MALDI tissue images [147], which can be combined with MRI imaging and histology to create an organ image, e.g., kidney [148]. Images can be constructed using MALDI, SIMS (analytes up to 1–2 kDa), DESI and LAESI, yielding a 3D SIMS image of HeLa cells, and MALDI mouse and rat brain images [149]. In a MALDI image of a glioma tumor in mouse brain, two tumor-associated proteins were localized to the MRI-imaged tumor [150], illustrating the potential for biomarker discovery in 3D.

The high resolution and mass accuracy of a Fourier transform ion cyclotron resonance instrument, combined with a high resolution-imaging source, allows single cell imaging [138] of metabolites and lipids, with accurate identification, and more specific images [138]. The use of ion cyclotron resonance or an orbitrap mass analyzer can overcome mass accuracy limitations due to samples of variable thickness in TOF analysis that is used for MALDI imaging [151].

Imaging MS is a useful approach to biomarker discovery for complex, spatially heterogeneous tissues, e.g., solid tumors, where principal components analysis and hierarchical clustering of MALDI imaging spectra can separate tumor tissue from non-neoplastic mucosa [152] and non-tumor, insulinoma and adenocarcinoma tumor types in pancreatic tissue [139]. MALDI imaging has been used for the analysis/diagnosis of several cancer types [153] and can locate gliomas and distinguish tumor grades, utilizing S100B protein [154]. A MALDI imaging-derived 4355 m/z MEKK2 peptide discriminated prostate cancer from normal tissue in tissue sections [155] while classifiers based on MALDI imaging of 171 patient samples distinguished six different adenocarcinoma types, and identified hepatic cancer and colon cancer metastasized to the liver [156].

Critical challenges for MS imaging include: optimization of sample preparation and cell fixation [131,149]; careful statistical analysis of the large data sets [157]; workflows for the analysis of 3D imaging data that are still under development [148]; and a tradeoff between tissue section imaging spatial resolution and analyte detection sensitivity [153]. When using a low-resolution instrument, more than one metabolite precursor ion can be included in MS/MS spectra, resulting in difficulty identifying analytes [158]. Also, as noted, some metabolites may not be in MS databases and cannot be identified using MS and MS/MS spectra, while ion suppression may limit detection of lower level peptides or metabolites and larger proteins (above 20–30 kDa).

3.2.6. Genome wide association studies (GWASs)

GWASs rely on screening a genome-wide set of hundreds of thousands to millions of SNPs for many individuals, and associating individual SNPs with disease phenotypes, thus defining genes associated with that disease [159]. As of August 2012, over 1700 GWAS studies have been completed on a variety of disease states [160]. Comparing allele frequencies of variants in thousands of individuals with and without disease can provide sufficient statistical power to detect alleles of small effect. However, with so many SNPs being assayed at one time, some may appear to cause an effect by chance, thus stringent *P*-values between $5e^{-7}$ and $5e^{-8}$ (or below) must be utilized for the results to be significant [161]. A more global evaluation of disease-associated genes involves next-generation (highly multiplexed) DNA sequencing (NGS) of individual human genomes. This form of sequencing is ~50,000-fold less expensive than the Sanger sequencing used for the human genome project [159]. Whole genome sequencing not only detects sequence variation in genes and noncoding DNA, but also copy number variants (CNVs) which are DNA insertions, deletions or rearrangements, e.g., inversions and translocations. Approximately 60,000 CNVs, 850 inversions and 30000 indels (an insertion or deletion less than 1 kb) have been identified in healthy individuals with several million SNPs differing from published DNA reference sequences [162]. Identification of functional DNA elements (e.g. transcription factor binding sites) in human DNA formerly categorized as "junk DNA" from the controversial ENCODE (Encyclopedia of DNA Elements) project, as well as tissue specific intergenic transcripts like lincRNAs (long intergenic noncoding RNAs [163]) have implications in the development of disease diagnostics [164].

GWAS has been utilized to examine genes associated with complex (non-Mendelian) diseases, e.g.,late-onset (>65 years old) AD (LOAD), with more than a dozen studies implicating the risk effects of 12 genes for late-onset disease [165]. Except for the apolipoprotein E ε4 allele, for which heterozygotes and homozygotes have 2.5-fold and 15- or 16-fold increased risk of LOAD, respectively [165,166], the risk effects of the other 11 genes, clustering to three pathways (immune system function, cholesterol metabolism, synaptic cell membrane processes), are small (e.g., allelic odds ratios 1.10–1.20 for increased risk for each altered gene [167]). Collectively the non-ApoE genes account for about 50% of LOAD genetics, with ApoE accounting for approximately 60–80% of the disease risk, the rest being due to environmental factors [165]. ApoE ε4 is thus a useful genetic biomarker for increased risk for LOAD. In addition to testing for multiplicity in GWAS studies, other potential issues include: (a) small risk effects of genes associated with the phenotype of interest; (b) potential differences between association and causation of phenotype, requiring additional functional experiments to examine causality; (c) incorrect mapping of marker SNPs; and (d) misassignment of genes in the human genome [168].

3.2.6.1. Next-generation DNA sequencing. Targeted resequencing of GWAS DNA segments using NGS [169] allows detailed mapping for the identification of high-risk alleles; exome sequencing allows complete coding region sequencing, while sequencing of the entire genome includes the exome as well as noncoding DNA, that can also be involved in phenotypes [159]. NGS has been used in Parkinson's disease (PD) [170], a progressive neurodegenerative disease involving slowness of movement, tremor, muscular rigidity and loss of dopaminergic neurons. Age is the major risk factor, and less than 20% of cases follow Mendelian inheritance; alterations in at least 5 genes have been associated with familial PD [171,172], while 16 PD gene loci and at least 8 genes have been identified as contributing to the sporadic disease [173,174]. Exome sequencing identified mutations in the VPS35 gene, (involved in cellular transmembrane protein sorting and recycling) in PD neurodegeneration [175] and mutations in SPG11 in juvenile PD, which also contribute to autosomal recessive juvenile amylotrophic lateral sclerosis (ALS) and hereditary spastic paraplegia [176,177]. NGS has also identified epigenetic changes in PD promoter DNA CpG hypermethylation, which controls protein expression via transcriptional silencing of gene expression [178,179]. NGS was used to identify DNA methylation differences in the promoter and first intron of α-synuclein (a major risk locus for PD) from different brain regions of PD patients compared to controls [180] which may be linked to overexpression. Other studies have identified altered brain α-synuclein DNA methylation in PD [181–183] but not in leukocytes (examined as a potential blood biomarker), consistent with an occasional lack of correlation of DNA methylation patterns between different tissues [184]. NGS has also been used in support of the hypothesis that β-secretase (BACE1)-mediated cleavage of amyloid precursor protein (APP) is involved in AD pathogenesis in an Icelandic population where a A673T coding mutation in APP adjacent to the BACE1 processing site protects against AD *and* cognitive decline in the elderly without AD [185]. These findings are consistent with Aβ peptides being biomarkers for AD [186–188].

4. Approaches to analysis

A standard method of analyzing biomarker performance, for a defined set of samples, is the ROC plot [189]. Using a binary diagnostic for cancer as an example, the plot *y*-axis is the true positive (correctly identified subjects with cancer) rate (sensitivity), and the *x*-axis is the false positive rate (subjects without cancer, incorrectly identified as having cancer) or 1- specificity (Fig. 1). The corresponding AUC is a measure of the diagnostic performance of the biomarker; an AUC of 0.5 indicates performance no better than chance, while an AUC of 1.0 indicates a perfect classifier. The AUC allows a comparison of the performance (when the analysis is done on the same sample set) of different biomarkers or biomarker panels, and presupposes a separate method (e.g., gold standard analysis, such as histopathology for organ toxicity) of accurately determining true positives. ROC plots have been proposed as part of "best practices" for biomarker qualification for the Predictive Safety Testing Consortium (PTSC) [190,191]. The Early Detection Research Network (EDRN) uses ROC plots as part of a verification process for each individual biomarker candidate, and then for panels of different combinations of individual biomarkers.

Along with biomarker sensitivity (the true positive [in this instance, diseased subjects] rate, or percent of positives correctly identified as such) and specificity (the true negative rate, or percent of negatives [non-diseased subjects] correctly identified), ROC AUC can be evaluated for each biomarker or panel of biomarkers. For specific contexts of use, the tradeoff between sensitivity and specificity, obtained from the ROC plot, may need to

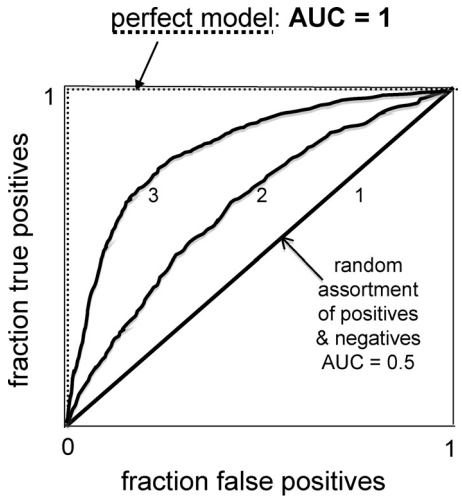

Fig. 1. Receiver operating characteristic (ROC) plot of true positive rate vs. false positive rate. A perfect model of biomarker data has an AUC of 1.0, while model 1, which separates positives from negatives no better than a random assortment, has an AUC of 0.5. The performance of model 3 is better than for 2, which is better than 1.

be explicitly defined. The required sensitivity and specificity for effective detection of a disease like cancer will vary with the type of cancer, its frequency in the population being screened, and the consequences of false positives (unnecessary medical procedures) and false negatives (failure to detect existing disease) [192].

For ovarian cancer screens in the general population, an upper limit for the false positive rate might be 2%, while a much higher false positive rate might be acceptable when the screening population is females with benign ovarian disease [193]. For 68 ovarian cancer biomarker candidates examined by the EDRN, only a combination of the existing marker CA125 and HE4 (now an FDA-approved panel for monitoring for recurrence of ovarian cancer) had adequate preclinical sensitivity (95%) and specificity (over 50%) in verification studies [193,194].

Another consideration involves estimating the number of subjects to be analyzed to detect a biomarker effect of a given size. This is critical for biomarker discovery and for clinical trial design; sample size determination is necessary to insure adequate statistical power for making inferences about a large population from a smaller sample [195]. Many biomarker studies have examined fewer than 100 specimens and lack the statistical power needed to associate a biomarker with a particular condition [1,195]. For a large number of samples, the sample size, n that needs to be examined to detect a biomarker difference, Δ in the population mean, μ to guarantee the statistical power $1-\beta$ is calculated according to equation 1. Here β is the type II error rate (the probability of failure to reject a false null hypothesis), the statistical power $1-\beta$ is the probability of correctly rejecting a false null hypothesis, and α is the type I error rate (the probability of rejecting a true null hypothesis). Usually the type II error rate β is at most 0.2 and statistical power is at least 0.80; α is commonly 0.05 [196].

$$n = \left[(Z_{\alpha/2} + Z_{\beta})\frac{S}{\Delta}\right]^2 \quad (1)$$

where $Z_{\alpha/2}$ is the z value corresponding to the right tail area of $\alpha/2$ in a normal distribution, and is equal to $[(\bar{X}-\mu 1)/(\sigma/(n)^{0.5})]$; $\bar{X}$ is the population mean, $\mu 1$ is the lower limit of the $100(1-\alpha)\%$ confidence interval, σ is the standard deviation, S^2 is the calculated sample variance, and n is the number of examined samples in a normal distribution. Z_{β} is the cutoff z value corresponding to the right tail area of β in a normal distribution. The statistical power and n are normally calculated from pilot studies, and are critical for clinical trial design [196].

Due to the complexity and size of datasets frequently evaluated for biomarker candidates, multivariate analysis is often utilized [197], but the details depend on the data structures being analyzed. When analyzing biomarkers from datasets with a large number of potential candidates, estimating the number that might strongly correlate with outcome by chance alone (multiplicity) is done using a false discovery rate [198]. For analysis of complex data such as from imaging MALDI (a dataset may contain 5000–50000 spectra, each with 10000–100000 m/z -intensity values, with spectra from different locations in 3-D tissue), unsupervised (applied without prior knowledge of the system) data mining methods are used for data reduction and include principal component analysis (reduces data dimensionality, visualizes most important influences on data variance [199]) or hierarchical clustering [160,200]. Supervised classification requires at least two classes to be differentiated (e.g., for a binary classifier, two classes could be no disease versus disease), utilizes a training set of data from known (in this example) diseased samples and known non-diseased samples to learn to discriminate the two groups, and is then applied to new data to determine their class [200]. The performance of the classifier can be analyzed using a test set of data not utilized in training the classifier; ROC plots can be used to summarize classifier performance. Examples of binary classifiers include the support vector machine, which has been applied in the analysis of MS-proteomics data [201] and proteomics-based biomarker analysis [202]; see Luts et al. for review [203]. Other classifiers include decision trees [204], random forests (sets of decision trees) [205] utilized to analyze ADNI serum and plasma protein biomarker data [206], and artificial neural networks, utilized to examine ovarian cancer biomarkers [207].

4.1. A single biomarker versus a biomarker panel

A key question in biomarker validation is whether a biomarker panel can outperform a single biomarker. In a number of cases a panel outperforms a single marker, but in others one or two markers may work well for defined goals. The use of multiple signaling pathways in transformed cells, as well as widespread tumor heterogeneity in the major epithelial cell-derived cancers [208], suggests that multiple biomarkers may more adequately reflect heterogeneous tumor biology and outperform single biomarker analysis [130]. Likewise, the presence of different confounding conditions in different patients may make accurate diagnosis more difficult, requiring a higher information content in a biomarker panel. However, adding additional markers may increase sensitivity at the expense of specificity. In the EDRN protocol developed for biomarker analysis, after blinded biomarker assay results from the established "reference data set" are obtained, individual biomarkers are examined for sensitivity and specificity. Then panels of biomarkers are constructed and examined using classifiers to see if biomarker performance can be improved beyond that of any single biomarker, which has occurred for prostate cancer (two markers to date, see below) and for ovarian cancer (panel of CA125 and HE4 for prediction of early recurrence, see below).

The OncotypeDx test for 10-year recurrence of lymph node–negative, estrogen receptor–positive breast cancer, is based on 21 genes, including 5 reference genes for normalizing expression of the 16 cancer-related genes, which are related to proliferation, invasion, HER2, and estrogen action [209]. The predictive power of the panel was greater than that of individual genes, while expression of single genes such as HER2 or the estrogen receptor were only weakly predictive of recurrence risk. The MammaPrint

test for lymph node–negative breast cancer survival of young women is based on human DNA microarray-determined [210] transcription levels of 70 genes, which were selected from 231 genes (of approximately 25,000 human genes examined) that were significantly correlated with disease outcome for this test (the appearance of distant metastases within 5 years after diagnosis) [211]. The 231 genes were rank-ordered based on their correlation coefficient to disease outcome, and groups of five genes were added to a supervised two-layer (estrogen receptor signature, BRCA-1 signature) classifier, including Monte Carlo simulations, until the combined false positive and false negative error rates reached a minimum, which occurred at 70 genes [212]. In this poor-prognosis signature, up-regulated genes were associated with the cell cycle, invasion and metastasis, angiogenesis and signal transduction.

AD is complex and heterogeneous in causation, including potential contributions from genetic polymorphisms such as ApoE variants, β-amyloid-related genes and Aβ42 peptide, pathogen infection, epigenetic modifications, or environmental insults [213]. Thus it may be expected that biomarker panels are preferred diagnostics. A panel of 10 serum autoantibodies distinguished AD from non-diseased controls with 96% sensitivity and 92.5% specificity in a small study of 50 AD patients and 40 controls; on average over 1000 autoantibodies were detected per AD serum sample [214], consistent with the suggested involvement of autoimmunity in AD [215], and autoantibody-involved pathogenesis [216]. A panel of 30 serum proteins, combined with ApoE allele identity and patient demographics, distinguished AD patients from controls with 94% sensitivity, 84% specificity and a ROC AUC of 0.95 [217]. CSF Aβ42 alone could be used to confirm autopsy-detected AD with a sensitivity of 96%, specificity of 76% and ROC AUC of 0.91 [187], although a classifier combining clinical scores and [^{18}F]-deoxyglucose imaging distinguished AD patients from controls with a ROC AUC of 0.97 [218]. Desmosterol, which is metabolized to cholesterol, is decreased in AD and as a biomarker has a ROC AUC of 0.80 [118], consistent with dysregulation of cholesterol metabolism in AD [219]. Thus for some cases a single biomarker may have utility as a diagnostic, but for complex diseases, panels of two or more markers appear to have superior performance.

5. Biomarker qualification and subsequent development

After biomarker discovery, subsequent development involves careful qualification and analytical validation, and validation in clinical trials. Due to the multidisciplinary complexity of the effort, such programs are "big science," involving consortia of multiple labs and organizations. Paradigms for all or parts of this extended process can be found in three large-scale biomarker initiatives.

5.1. Predictive Safety Testing Consortium (PSTC)

The PSTC [220], a collaboration between the FDA and the Critical Path Institute, was intended to discover and develop biomarkers for drug safety, including preclinical and clinical kidney toxicity, liver toxicity, skeletal muscle myopathy and cardiac hypertrophy, testicular toxicity, vascular injury and carcinogenicity [221,222]. Qualified biomarkers were intended for use in predicting a range of toxicities frequently observed in drug development and in early clinical studies [223]. Here "biomarker qualification" required data that critically assessed the analytical and biological performance of the biomarker, including comparison to the existing "gold standard". For renal toxicity these data included the use of a validated analytical assay for the biomarker; the biological plausibility of the biomarker association with renal toxicity; a molecular level understanding of the mechanism of biomarker response; a strong correlation between biomarker changes and renal pathology; improved performance relative to currently accepted biomarkers; a consistent biomarker response across mechanistically diverse toxicants, subject sexes, and animal species; dose- and temporal-response linking the magnitude of biomarker changes to the severity of the organ injury and the onset/recovery from injury; identification of early kidney injury; specificity for kidney (versus other organ) injury; and biomarker accessibility in serum or urine [223].

The study group selected 7 biomarkers from 23 candidates for qualification, and based on the performance of individual biomarkers in rat toxicology studies, proposed, when used alone or in combination with existing markers (serum creatinine, blood urea nitrogen content), that single markers or defined combinations be used in rat GLP (Good Laboratory Practice) studies supporting early clinical trials, as early diagnostic markers for onset (but not regression) of drug-induced acute kidney tubular or glomerular alterations resulting in impairment of tubular reabsorption [190,223,224]. The markers have not so far been approved by the FDA for use in human clinical trials for these purposes, but their voluntary use as evidence additional to traditional biomarkers and histopathology in rat studies was allowed [190]. Their use in human clinical trials to gather additional data supporting their usefulness as renal toxicity biomarkers may be considered on a case-by-case basis [190].

5.2. Early Detection Research Network (EDRN)

The National Cancer Institute EDRN focused on biomarkers for early detection of cancer [194,225], which can be difficult as early stage tumors are small and biomarkers shed into blood can be present at low levels [208]. Three hundred biomarkers were prioritized with procedures for serum and plasma collection being critical [74,226]. Samples were annotated using the NCI Biospecimens First Generation Guidelines [194]. Five phases of biomarker development were proposed, the first being preclinical exploration/discovery of biomarkers. Most of the biomarker candidates did not progress beyond this phase either due to the small changes observed in cases versus controls or due to substantial variability in the control population [208]. Subsequent phases involved development of a robust and reproducible clinical biomarker assay [226,227] where many biomarker candidates failed due to insufficient sensitivity or specificity. Of 300 EDRN biomarker candidates prioritized for additional study, 10 validation trials were completed, resulting in 5 FDA-approved biomarkers for various clinical endpoints, e.g., the use of the combination of cancer antigen 125 and human epididymis protein 4, for monitoring the early recurrence of ovarian cancer [194].

An example of the process focused on 108 biomarker candidates used with prostate-specific antigen (PSA) to improve prostate cancer screening. Only 58 of these gave reproducible results, and five were selected for further validation [63]. Gene expression profiling analysis of prostate cancer RNA identified chromosomal translocations of ETS (erythroblastosis virus E26 transformation-specific) transcription factors in ~50% of prostate cancer patients, with fusion of the ERG (v-ets erythroblastosis Virus E26 oncogene homolog) transcription factor with the androgen-responsive promoter, TMPRSS2 (transmembrane protease, serine 2) representing ~90% of ETS fusions occurring in prostate cancer [228,229]. The gene fusion was both tissue-specific and cancer-specific, and was involved in cancer progression. To overcome the lack of sensitivity of this single biomarker, it was combined in a panel with other biomarkers, e.g., the long noncoding RNA PCA3 [230], overexpressed in over 90% of prostate cancers [231]. The ROC plot AUC of the combined markers, both measured in urine, of 0.71–0.77 was better than either alone and

also better than the standard diagnostic PSA (ROC AUC = 0.60–0.61) [232].

The EDRN has also initiated examination of circulating tumor cells (CTC) as potential biomarkers. These reflect formation of metastases and are potentially useful for prognosis of melanoma [233], lung [234], ovarian, breast, prostate, colon and other cancers helping guide therapy of individual cancer patients as "liquid biopsies". They have been integrated into over 400 clinical trials [235]. Due to their genetic instability and a flexible genetic response to therapy or tumor microenvironment, tumors can be heterogeneous within a single patient, differing in gene expression and properties between the primary tumor and metastatic sites, or even within a single tumor at a primary or metastatic site [293]. Thus CTCs may also be heterogeneous. This has been observed for HER2 status, where discordance between the primary tumor, CTCs and disseminated tumor cells has been observed in breast cancer patients [294]. In a separate study, a subset of patients with advanced breast cancer had HER2-negative primary tumors but HER2-positive CTCs, suggesting this change may occur during tumor progression [295]. Thus use of CTC properties as biomarkers should be carefully studied.

5.3. Alzheimer's Disease Neuroimaging Initiative (ADNI)

The ADNI [236–238] is a longitudinal study, initiated in 2004, of 200 elderly controls, 200 subjects with mild cognitive impairment (MCI), 400 subjects with mild-to-moderate AD, and (more recently) 100 subjects with early MCI, to develop biomarkers for early (pre-dementia) AD diagnosis for tracking disease progression, to support clinical trials, and in a secondary phase (ADNI-2) to identify subjects at risk for AD [238]. Potential biomarkers included neuroimaging, using [^{8}F]-deoxyglucose (FDG) and [^{18}F]-florbetapir and MRI structural imaging to measure changes in glucose, amyloid deposition and brain volume, respectively [239], genetic profiles, and blood and CSF biomarkers including Aβ42 peptide and tau protein [238].

The five most studied AD biomarkers - CSF Aβ42, CSF tau, amyloid-PET and FDG-PET imaging and structural MRI imaging were examined [240] as a function of cognition using the MMSE (Mini-Mental State Examination [241]). The time courses were complex and deviated from baseline values as the MMSE scores worsened. Evaluation of an AD progression score (ADPS), derived from seven AD biomarkers [hippocampal volume, CSF Aβ42, CSF tau, MMSE score and three other measurements of cognition/dementia, namely ADAS (Alzheimer's disease assessment scale), CDRSB (clinical dementia rating sum of boxes score) and RAVLT 30 (Rey auditory verbal learning test 30 min recall)] fit a sigmoidal function with the temporal order of biomarker changes (Fig. 2) being similar to those reported by Jack et al., [242], with the exception that the RAVLT30 change preceded changes in all the other biomarkers [245]. Changes in the time course of four biomarker classes (CSF Aβ1-42, CSF tau, neuroimaging data [hippocampal volume, brain ventricular volume, FDG-PET] and memory (RAVLT30) and executive function (ADNI-EF (executive function) test) during AD progression supported the hypothesis that earlier biomarker changes [244] had a causal role in later changes, with the exception that CSF Aβ42 and CSF tau changes were independently associated with brain structural and functional changes, and changes in CSF tau and brain neuroimaging had an independent relationship with cognition [246]. The time course of biomarker changes in familial AD patients (representing 1% of the AD patient population) showed that decreased CSF Aβ42 began 25 years before symptom onset. β-amyloid deposition, CSF tau, and brain atrophy began 15 years before expected symptoms, and cerebral hypometabolism and impaired episodic memory began changing 10 years before symptom onset. Cognitive impairment,

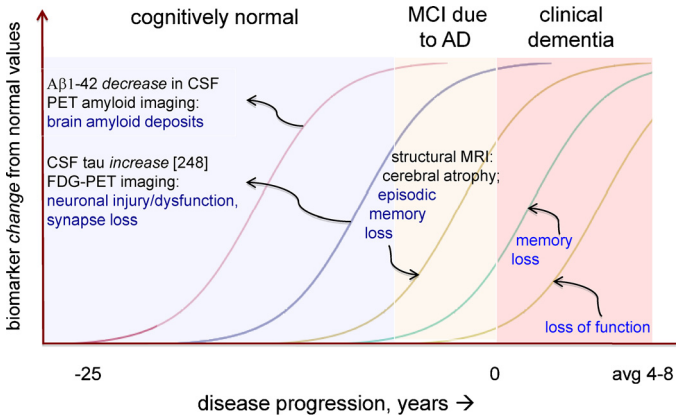

Fig. 2. One model of AD progression based on [242]. Biomarker types are in black text; functional changes are in blue text. Different biomarkers change at different times during disease progression; this can vary with different patients. CSF Aβ1-42 levels may begin decreasing 25 years before the onset of symptoms in dominantly inherited AD [243]. Progression includes 3 stages: a period of normal cognition, MCI, and dementia, followed by death an average of 4–8 years later [244]. Combinations of biomarkers can be used to help define the stage of disease for individual patients, and may allow stage-targeted treatments in the future. ADNI datasets have been useful for testing this model [240,245,246]. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

measured by MMSE score and the CDR scale, was detected 5 years before symptom onset, with patients meeting diagnostic criteria for dementia 3 years after symptom onset [243]. It remains unclear that these results are applicable to patients with sporadic AD. ADNI biomarker data has also been used to study the transition from MCI to AD [247–250].

6. Biomarker validation

Beyond biomarker assay validation [3], biomarker validation is intended to address whether a biomarker achieves its purpose in a carefully defined clinical setting and population of interest, better than the current gold standard [2,4,63]. For a diagnostic biomarker, does it predict disease in (often asymptomatic) individuals? For a prognostic biomarker, does it predict disease progression or outcome in an untreated individual? For a predictive biomarker, does it predict patient benefit or toxicity from a specific therapeutic intervention?

The predictive nature of the biomarker, which may be added to an existing panel to improve performance, is addressed in terms of sensitivity and specificity (see [251]) in multiple populations. Cross-validation studies by two or more groups independent of the one submitting initial studies for FDA review have been proposed [252].

Clinical trial design is important for biomarker validation, and can be complicated by the heterogeneity of human disease [253]. For prospective randomized case-control clinical studies, standards for diagnostic or prognostic biomarker study design (e.g. for patient and control subject selection) confounding variables, study performance criteria and study size are discussed [192]. Matsui [254] discusses biomarker clinical validation and clinical utility criteria, including randomizing patients on standard-of-care or on new biomarker-determined treatments. In enrichment designs, appropriate when patients without the biomarker likely will receive no benefit from the new treatment, biomarker-positive patients randomly receive either the new treatment or a control treatment. In a randomize-all or all-comers design, appropriate when there is no strong evidence that a new biomarker predicts treatment effects, patients are randomized with respect to the presence of the biomarker candidate, or stratified based on the

presence of the predictive biomarker [255]. In adaptive designs, that are useful when multiple treatment options or multiple biomarker-defined subgroups exist, clinical trial parameters are modified during the trial based on accumulated data [255].

7. Challenges

Additional challenges to biomarker development include the following.

7.1. Most published biomarker candidates do not enter clinical practice

The vast majority of candidate biomarkers are eventually abandoned [63,69,256–257]. In 2010 over 20,000 papers documented biomarker investigations, including 8000 potential cancer biomarkers, 600 of which were thought to be useful for early detection [63]. To date, relatively few (ranging from 0–2 per year from 1996–2003) newly-characterized plasma protein biomarkers (for example) have been approved [258]. From an estimated 150,000 papers documenting thousands of claimed biomarkers, fewer than 100 have been validated for routine clinical practice [1].

The factors that contribute to the attrition of biomarker candidates include:

(i) the need for sustained efforts to translate initial research results to FDA approval [63];
(ii) the need for "big science" to analyze and develop biomarkers for complex and heterogeneous diseases e.g., cancer or AD [1,259];
(iii) an effective team process [256,260] with collaborations from multiple laboratories to standardize protocols, examine biomarker reproducibility, independently analyze data, conduct independent validation [69] and large scale (e.g. randomized) clinical trials [261];
(iv) bias, e.g. from inappropriate study population selection, the methodology/technology used for analysis [98,256] or from data overfitting [262].

Early biomarker candidates from individual labs have often been fragmented and disconnected from subsequent validation and analysis with assays often not being reproducible and having questionable generalizability [258], with most biomarker candidates not progressing beyond the discovery stage due to insufficient sensitivity or specificity [208].

7.2. Problems with selection of preclinical samples

The selection of preclinical samples used for biomarker discovery, for example for screening/early detection of ovarian cancer, is critical [263]. While many biomarkers have been proposed in this case, the gold standard remains CA125, a membrane-associated mucin family glycoprotein. In two studies [264,265] comparing CA125 with other candidate biomarkers and biomarker panels, the inclusion of samples from symptomatic patients, including those with advanced disease, was problematic in view of the need to screen for biomarkers to detect disease in advance of symptoms, with samples ideally being collected 6–12 months prior to cancer diagnosis. A comparison of results from unblinded samples obtained at the time of clinical diagnosis, with blinded samples acquired prior to clinical diagnosis, suggested that systematic bias led to prior exaggerated reports of biomarker performance [263], and suggested that the PRoBE "prospective-specimen-collection-retrospective-blinded-evaluation" protocol [192] could circumvent such problems.

7.3. Sample storage and handling

Biomarkers and their analysis can be affected by differences in sample storage time and conditions, e.g., a negative correlation of SELDI-TOF peak intensities with time of storage [106]. Repetitive freeze-thaw cycles can have a dramatic effect on enzyme activity [266], seminal plasma protein levels [267], and protein denaturation [268,269]. Sample collection should thus follow a rigorous protocol, including the documentation of factors that might influence biomarker values (e.g., freeze-thawing), and of the diagnosis associated with the sample. Samples from multiple centers should be analyzed and sample blinding and randomization should be implemented [226].

7.4. Pooling samples

While pooling individual samples prior to analysis can shorten analysis times and generate more total sample amount for a single analysis, interindividual covariance and variation in biomarker candidate levels can be lost or minimized. Detection of outliers and misclassified samples can also be challenging, and transcript changes identified in individual samples may not be identified in pooled samples [270]. Data can also be lost in proteomics experiments, with 50% of SELDI-TOF peak clusters detected in individual samples being lost after pooling, with the loss being greatest for low-intensity peaks, potentially limiting detection of lower level biomarker candidates [271]. With simulated gene expression data, increased sample pooling also increased prediction error [272].

7.5. Biomarker dynamic range

In plasma or serum proteomics, proteins exist in a dynamic range of ~12 orders of magnitude and are dominated by ~22 abundant proteins, which make up approximately 99% of the total protein mass [273], making the detection of lower abundance proteins difficult. One approach to solving this problem involves chromatographic immunodepletion using immobilized antibodies raised against the most abundant 7–14 proteins, and optionally against ~50 medium-abundance proteins, for which tested columns appear reproducible [274]. However in one report, non-targeted plasma proteins were enriched an average of approximately 4-fold by the top-14 immunodepletion, and the 50 most abundant remaining proteins accounted for approximately 90% of MS/MS spectral counts, leaving a restricted capacity for examination of lower abundance proteins for biomarker candidates [275]. This suggests the need for additional fractionation (and likely lower overall throughput).

For hypothesis-driven (targeted) protein biomarker discovery, selective reaction monitoring of predefined (proteotypic) peptides from targeted proteins may allow examination of lower level biomarker candidates [276]. In light of the distinct possibility that low abundance proteins (which may include specific biomarkers) may bind to column resins, immobilized antibodies or immobilized antibody-antigen pairs, all fractions of the columns need to be examined.

Plasma or serum biomarker discovery studies may not include immunodepletion to simplify and speed up the analysis. The tradeoff is that specific markers of particular disease processes that are present in low relative abundance are greatly diluted [2] while identified biomarker candidates in unfractionated plasma may include a number of relatively abundant acute phase response proteins, e.g., albumin, transferrin, serum amyloid A, serum amyloid P, haptoglobin, α2-macroglobulin, fibrinogen, α-1-acid glycoprotein and ceruloplasmin. Subsequent to the acute phase response, complement activation, protease inhibition, clotting,

opsonization and other changes occur [277]. Over 200 acute phase proteins exist [278,279], are part of the innate immune response, and are activated by trauma, infection, stress, cancer, and inflammation [277]. Acute phase proteins and proteins downstream from this process are thus highly unlikely by themselves to be specific biomarkers.

7.6. Data analysis and bias

7.6.1. Batch effects

Batch effects are groups of measurements that have qualitatively different behavior across conditions unrelated to biological variables in a study [280]. They are widespread, may be larger than a biological effect, and occur when biomarker data collection is affected by operational issues, e.g., personnel changes, reagent lots, lack of instrument calibration, misalignment of data in some but not all datasets, different sample processing, or changes in lab conditions that lead to incorrect conclusions [280,281]. In such instances, upon principal components analysis or clustering, samples might cluster by time, processing group [280] or another non-biological variable. Batch effects have been implicated for example in analysis of ovarian cancer proteomic patterns [105] and in the use of microarrays to obtain genomic signatures for ovarian cancer [282]. Batch effects can be corrected by careful study design (e.g. randomizing biological groups, such as cases and controls, across batches).

7.6.2. Selective data reporting, incomplete validation and forensic bioinformatics

Selective reporting or non-reporting of data can bias the reported strength of biomarkers [283]. A meta-analysis of 18 studies on the use of the tumor suppressor p53 as a prognostic factor for head and neck squamous cell cancer found that when 13 additional published studies were included, as well as unpublished data retrieved directly from investigators, the statistical significance of the association between p53 status and mortality disappeared [283]. Data and analysis errors can also cause problems. The use of gene expression signatures to predict response to chemotherapeutic drugs [284] was subsequently retracted [285] due to incomplete documentation of the data used for analysis. The analysis process itself obscured numerous errors that included errors in data labeling, such as reversal of cell line labels as drug-sensitive or drug-resistant in numerous cases, incorrect labeling of test data, analysis of genes for which no microarray data existed, off-by-one gene indexing errors, batch effects due to use of different array scanners with different datasets, use of three different rules for calculating the probability of sensitivity to a particular drug treatment, and mistakenly assigning the gene signature for one drug to another drug [286]. Solutions from this forensic bioinformatics analysis of high-dimension data include the proposal to supply all raw data, the computer code used for analysis, exact instructions for each step of data analysis, and labels for all of the experiments being analyzed, to allow an independent investigator to reproduce each step as well as the final results of the analysis [287]. Inadequate validation can also bias results. Examination of the seven largest published DNA gene expression microarray studies by inclusion of multiple random test datasets observed that genes identified as predictors of cancer outcome were highly unstable, with gene signatures depending on patient selection in the training sets. Five of the seven studies did not classify patients better than chance [288].

7.7. Study design

Issues with study design can also bias study outcome [69]. For one study of exoprotease-generated peptide patterns for diagnosis of prostate cancer, cases were all males while a large fraction of controls were females; the average age of cases was 60 years, while the average age of controls was 40 years, at which age prostate cancer is rare [69]. Another study reported that plasma lipoprotein lipid (including triglycerides) proton NMR line widths could distinguish a variety of cancers from non-cancer control subjects [289]. However plasma lipid composition is associated with age, sex and diet, none of which were matched between cases and controls. When cases and controls were matched for age and sex, the distinction between patients with and without cancer disappeared [290,291].

8. Conclusions

Effective biomarkers exist in clinical practice, e.g., CA125 for early diagnosis of ovarian cancer, serum creatinine as an indicator of renal function, and elevated serum cardiac troponin as an indicator of cardiac injury [69], but their development has required many years and a large scale, multidisciplinary effort. Fewer than 1% of published cancer biomarkers, enter clinical practice [256].

Many factors can contribute to the failure to validate the clinical utility of a published biomarker: optimized standard operating procedures for a variety of steps may not be in place; clinical samples studied for biomarker discovery or validation may not be appropriate for the disease being examined; and assays used for validation may not be reproducible, tested externally in independent labs, or on independent samples.

Compared to biological variability in target clinical populations, only small biomarker differences may exist between cases and controls. Clinical studies may have inadequate statistical power to demonstrate biomarker utility. Statistical analysis may be insufficient. Bias from many sources may not be adequately controlled. Data analysis may not be systematic or reproducible and may contain many errors. Information sufficient to allow an outside investigator to completely reproduce data processing and analysis results, including computer code and raw data, may not be available, and this may obscure problems with data analysis. Care needs to be taken in *all* of these areas as problems in a single area can compromise the validity of biomarkers that have taken years to develop.

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