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Overview of Targeted Quantitation of Biomarkers and Its Applications

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1.1 Introduction

In the last two decades, utilization of biomarkers in drug discovery and development has seen rapid growth as a result of the advancement of laboratory techniques and bioanalytical assays including ligand-binding assays (LBA) such as enzyme-linked immunosorbent assay (ELISA), quantitative polymerase chain reaction (qPCR), and mass spectrometry (MS)-based technologies, and so on (Anderson and Kodukula, 2014). Currently, pharmaceutical companies and regulatory authorities are actively engaged in developing robust efficacy and safety biomarkers that can be used in a translational manner to assist drug development by making the right choice for "go" and "no-go" decisions at the earliest possible stage. In order to most efficiently utilize the resources and maximize the benefits of biomarker research, most of the drug companies have established internal biomarker research centers and are also pursuing extensive collaborations with academia, hospitals, and research institutes. A biomarker can assist target and candidate selection in drug discovery, toxicity assessment, dose selection, and pharmacokinetics (PK)/pharmacodynamics (PD) modeling in drug development. In clinical Phase I–IV, a biomarker can help in patient stratification, drug–drug interaction (DDI) evaluation, efficacy assessment, safety monitoring, and companion diagnosis as well as postapproval surveillance. Biomarkers measured in patients before treatment can also be used to select patients for inclusion in a clinical trial. Changes in biomarkers following treatment may predict or identify safety problems related to a candidate drug or reveal a pharmacological activity that is expected to predict an eventual benefit from treatment. Biomarkers can also be used as diagnostic tools for the identification of population with an underlying disease and its progressive stage.

In fact, most of the drug programs in development stages have requirements of biomarkers to be incorporated in the preclinical and clinical development strategy as they can help ensure safety and efficacy of the drug candidates. Indeed, it has been reported that the ability of biomarkers to improve treatment and reduce healthcare costs is potentially greater than in any other area of current medical research (Drucker and Krapfenbauer, 2013). A search of one major clinical trial registry on December 5, 2015 (https://ClinicalTrials.gov), using the search term "biomarker," generated 17,366 results, almost twofold increases, compared to what had been previously reported 5 years ago (Boulton and Dally, 2010). Less than a year late (November 8, 2016), this number is 19,611.

More specifically, biomarkers have demonstrated the added values to every major disease area. For example, in oncology, with the growth in numbers of targeted therapies for oncology clinical testing, biomarkers are often used to select patient population (Arteaga, 2003). Biomarkers can also allow investigators to stratify patients for prospective or retrospective evaluation of different clinical responses and for identification of specific responder sub-population (Mendelsohn and Baselga, 2003). A previous publication also proposed optimizing oncology drug development by using a tiered set of clinical biomarkers that predict compound efficacy with increasing confidences as well as increasing rigor of validation at each of the three levels (Floyd and McShane, 2004). Level-1 biomarkers confirm biochemical or pharmacological mechanism of action by showing that the drug is modulating its target and provides correlation of PD and PK, which is the exposure of the drug and its active metabolites. Level-2 biomarkers confirm that the drug is producing a desired PD effect directly related to its potential for efficacy such as altered downstream cell signaling in pathways related to target, decreased metabolic activity,

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or changes in tumor vascular perfusion. Level-3 biomarkers have predictive power for a desired outcome and may be surrogate end points for in vivo symptoms, such as tumor size. It should be noted that even with the extensive research by many scientists over the last decades, very few biomarkers, that can be measured in the laboratory, qualify for Level-3 biomarkers. Of course, this type of categorization of biomarkers can also be applied to other disease areas. Almost all of the biomarkers discussed in this book belong to the first two levels.

For Type 2 diabetes (T2DM), it was estimated that, in 2010, 285 million people had been diagnosed with diabetes mellitus worldwide, a prevalence of 6.4% of the total population. This is predicted to increase to 439 million (7.7% of total population), and by 2030, T2DM will account for about 90% of diabetic patients worldwide (Shaw et al., 2010). Biomarker search has lead to several promising biomarkers such as Chitinase-3-like protein 1 (CHI3L1) also known as YKL-40, soluble CD36 (cluster of differentiation 36), leptin, resistin, interleukin 18 (IL-18), retinol-binding protein 4 (RBP4), and chemerin that could be indicative for the pathogenesis of insulin resistance and endothelial dysfunction in T2DM patients (Qhadijah et al., 2013). In another paper (Lyons and Basu, 2012), it was postulated that in blood, hemoglobin A1c (HbA1c) may be considered as a biomarker for the presence and severity of hyperglycemia, implying diabetes or prediabetes.

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills, and eventually the ability to carry out the simplest tasks. In most people with AD, symptoms first appear in their mid-60s. Estimates vary, but experts suggest that more than five million Americans may have AD (https://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-fact-sheet). There is significant interest in the development of methods to validate novel biomarkers for diagnosis of AD. Cerebrospinal fluid (CSF) levels of β-amyloid Aβ1–40 and Aβ1–42 peptides, total Tau protein, and phosphorylated Tau protein have diagnostic values in AD (Chintamaneni and Bhaskar, 2012). Tau protein is a highly soluble microtubule-associated protein (MAP). In humans, these proteins are found mostly in neurons compared to non-neuronal cells. Tau protein and phosphorylated Tau protein are measured by using ELISA (Herrmann et al., 1999). Liquid chromatography in conjunction with mass spectrometric detection (LC–MS)-based assays have also been published for measuring β-amyloid Aβ1–40 and Aβ1–42 peptides in CSF (Choi et al., 2013). A systematic review and meta-analysis of the literature on whether or not CSF total tau, phosphorylated tau, and β-amyloid Aβ1–42 peptide help predict progression of mild cognitive impairment to AD was conducted (Diniz et al., 2008).

1.2 Biomarker Definition

It is generally accepted in the pharmaceutical industry that a biological marker or a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathologic processes, or biological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). Biomarkers are typically classified into diagnostic, prognostic, and predictive biomarkers. Biomarker definition and usage are summarized in Appendix II of the Guidance for Industry and FDA Staff (Qualification Process Working Group, 2014).

A *diagnostic* biomarker is a disease characteristic that categorizes a person by the presence or absence of a specific physiological or pathophysiological state or disease.

A *prognostic* biomarker is a baseline characteristic that categorizes patients by degree of risk for disease occurrence or progression of a specific aspect of a disease.

A *predictive* biomarker is a baseline characteristic that categorizes patients by their likelihood of response to a particular treatment relative to no treatment.

In pharmaceutical industry research and development (R&D), biomarkers can also be described as efficacy or safety biomarkers. Division of common biomarkers into these two categories is probably better linked with the drug discovery and development process as deficiency in safety or efficacy is the major reason for termination of drug candidates. Efficacy biomarkers emphasize on mode of action and can be used to build early confidence in drug mechanism and can potentially substitute for clinical symptoms as a measurement of efficacy. Safety biomarkers are early markers of reversible or irreversible drug-induced adverse events and can be used to understand the mechanism of drug-induced toxicity.

An emerging area of extensive research is to use endogenous biomarkers to predict potential cytochrome P450 or transporter-mediated DDI. They can be used to assess changes in drug metabolism and transport phenotype due to intrinsic and extrinsic factors. The benefits of endogenous DDI biomarkers include better PK/PD correlation due to samples collected at multiple time points; resource sparing because it is a secondary objective in a Phase I clinical study; its applicability to studies in all population; its early signal for metabolic liability without conducting a separate clinical DDI study using exogenous drugs, such as midazolam, as probes.

It is very hard to identify and validate a good DDI biomarker. This process takes a lot of research and verification as well as extensive collaboration from multiple institutes, hospitals, and consortia to confirm the initial finding. For example, bioanalysis of 4β-hydroxycholesterol in human plasma is currently being proposed by Innovation and Quality (IQ) consortium as a potential

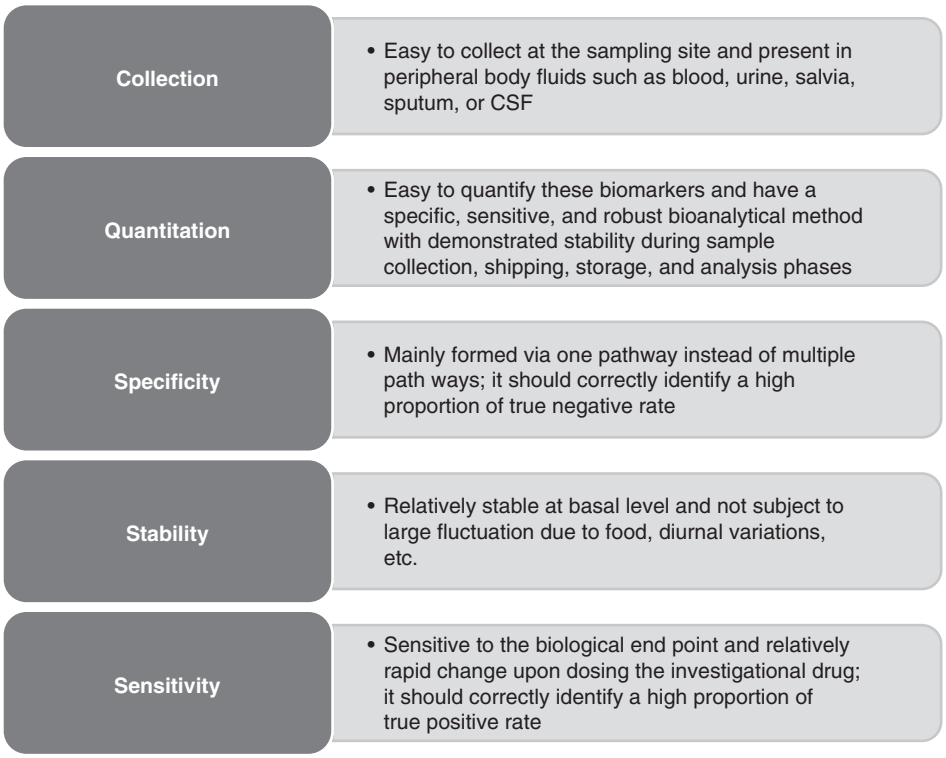

Figure 1.1 Features of ideal endogenous biomarkers on aspects of sample collection, quantitation, specificity, stability, and sensitivity.

endogenous biomarker for CYP3A4 induction (Aubry et al., 2016) even though the initial observation was made a decade ago (Bodin et al., 2001).

Ideal endogenous biomarkers including DDI biomarkers should possess the following features on sample collection, quantitation, specificity, stability, and sensitivity (Figure 1.1). Using 4β-hydroxycholesterol as an example, this biomarker meets essentially all of these features when used as a probe for CYP3A4 induction studies. The long half-life of 4β-hydroxycholesterol results in small variations in concentrations but excludes this marker in short-term studies. On the other hand, 6β-hydroxycortisol/cortisol ratio in urine, another frequently used endogenous biomarker for CYP3A4 inducer, is a more rapid biomarker due to short half-life with little delay time behind the changes of CYP3A4 activity in vivo. However, the short half-life and diurnal effect lead to more variable data, even with the correction in cortisol concentration (Dutreix et al., 2014).

1.3 Current Challenges of a Biomarker

The ultimate goal for a biomarker is the establishment of clinical utility that guides patient care, but attempts to reach this goal must be preceded by analytical and clinical validation of the "locked-down" biomarker assay. Even though endogenous biomarkers could become a valuable tool to assess liability early in drug development, nevertheless, out of thousands of biomarkers discovered through metabolomics and proteomics approaches, only a few dozens were found to be useful in assessing efficacy and toxicity of the drug candidates. The drugs may have an impact on multiple pathways of endogenous biomarkers' disposition and formation, and how to extrapolate a biomarker from healthy volunteers to patient population can also be a challenge (Drucker and Krapfenbauer, 2013).

Major challenges regarding integrated and harmonized processes, spanning preanalytical, analytical, and postanalytical phases of development remain (de Gramont et al., 2015). During biomarker development, robust laboratory methodology is essential at all analytical phases. Lack in biomarker characterization and validation by using robust analytical techniques, which is a lengthy process requiring careful planning and execution of assay development and validation, have been attributed to be major reasons. Problems with the collection, equipment, or transportation of specimens to the laboratory can affect the measurement of the biomarker. Improper storage of samples or changes in storage environment can also affect measurement of biomarkers (Mayeux, 2004).

1.4 Biomarker Validation Process

Over the last a few decades, drug discovery and development have been driven, in all therapeutic areas, by the pharmaceutical companies to become more productive and to launch more products onto the market in a cost-effective manner. Even with much investment in genomics, proteomics, metabolomics, and other "omics," the approved rate of New Drug Applications (NDAs) remains relatively flat. Majority of compounds entering clinical trials fail and many new approved products have significant labeling restrictions. In 2004, FDA's Critical Path Initiative (CPI) recognized that the process of drug development and the availability of new therapies were not fully benefitting from the many advances in biomedical science. In addition, drug development had become increasingly challenging and resource intensive. An important area identified by the CPI as potentially enabling significant progress in drug development was applying those scientific advances as new tools to aid the development process. Such tools could speed the availability of new products that may be safe and effective. FDA has undertaken multiple initiatives to support the development of new drug development tools (DDTs). Among these efforts has been the creation of a formal qualification process that FDA can use when working with submitters of DDTs to guide development as submitters refine the tools and rigorously evaluate them for use in the regulatory process. Because of the tremendous potential of biomarker utilization, it has been listed, along with clinical outcome assessments, and animal models, as the established qualification programs under DDT guidance.

The European Medicines Agency (EMA) also pays close attention to research in the use of biomarkers in the development of medicines. In August 2014, EMA issued a draft concept paper outlining the key elements to be developed in a guideline on good genomics biomarker practices. This is expected to facilitate the use of genomic data for the development of the so-called "personalized medicines," the safety monitoring of medicines, and the early diagnosis of disease.

Value of biomarker measurement that can characterize baseline state, a disease process, or a response to a treatment is well recognized by both agencies. A Joint FDA/EMA Letter of Intention (LOI) submission for biomarkers and clinical outcome assessment qualification programs was issued. Parallel submissions for qualification of biomarkers to both agencies are encouraged and both FDA and EMA will share their scientific perspective, advice, and response letters for the submission. With the joint LOI, the agencies intend to reduce the time taken by applicants to prepare LOIs. A joint EMA/FDA report on kidney injury was issued and a number of biomarkers including albumin, β2-microglobulin, clusterin, cystatin C, kidney injury molecule 1 (KIM-1), total protein, and trefoil factor-3 were recommended. Discussion on biomarkers for oncology and AD were also extensively researched. For example, β-amyloid 42 and total Tau protein in CSF were recommended as useful biomarkers by EMA. For drug-induced cardiotoxicity biomarkers in preclinical studies, cardiac troponins T (cTnT) and I (cTnI) in serum/plasma were proposed as biomarkers. In 2015, three clinical biomarkers were proposed by FDA: Fibrinogen in plasma as a prognostic biomarker for enrichment of clinical trials in chronic obstruction pulmonary disease (COPD); an imaging biomarker measuring total kidney volume (TKV) as the prognostic biomarker for enrichment of clinical trials in autosomal dominant polycystic kidney disease; and galactomannan as a serum/bronchoalveolar lavage fluid biomarker for patient selection for enrollment in invasive aspergillosis (IA) clinical trials.

1.5 Current Regulatory Requirement for Target Biomarker Quantitation

Biomarkers were typically discovered via metabolomics, proteomics, system biology, or their combinations. Bioanalytical assay development and validation will be used to support retrospective pilot and prospective pilot studies. The bioanalytical assays which play pivotal roles to support all phases of biomarker validation and "lock-down" for clinical usage should have the appropriate quality to allow good, robust, and data-driven scientific decision to be made. Sensitivity analysis is then performed to find the specificity of the biomarker to the biological end point. Finally, a well-designed and controlled validation study is conducted to finally confirm the utility of the biomarker. In these two late phases, well-established bioanalytical assays play even more significant roles since the assay performance can impact not only the outcome of the sensitivity test but also the numbers of subjects and samples that have to be used to establish utility of the biomarkers. A well-established bioanalytical assay with excellent robustness and precision is essential for biomarkers, especially when its level of change from predose to postdose is small.

Currently, fit-for-purpose assay establishment was proposed for biomarker assays (Lee et al., 2006). In brief, fit-for-purpose biomarker assay validation can be separated in four continuous iterative activities (Figure 1.2). This approach was then endorsed and adapted by both the regulatory authorities (Booth, 2011; Valeri et al., 2013) and other industrial organizations such as European Bioanalytical Forum (EBF) (Timmerman et al.,

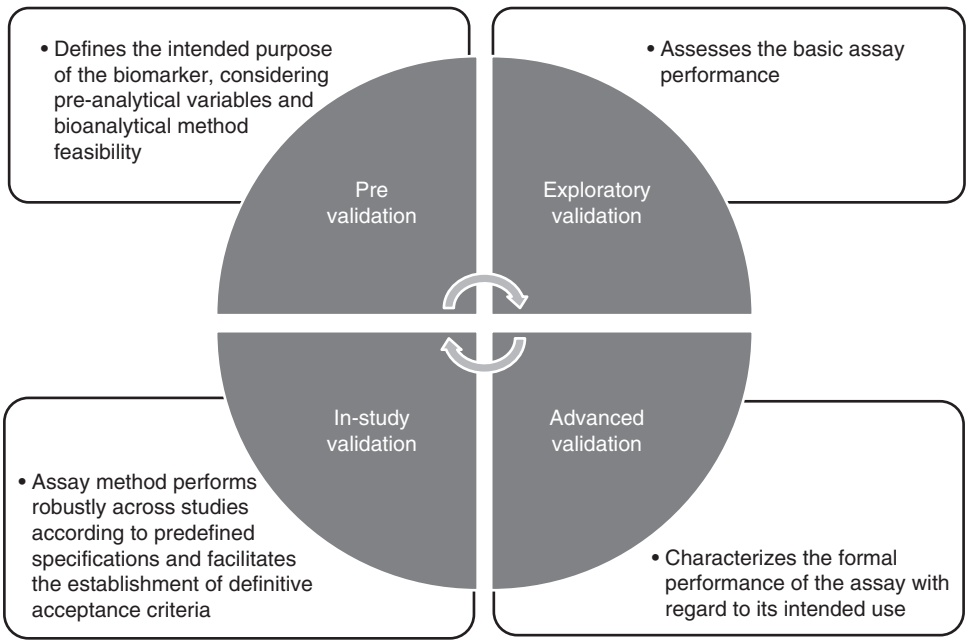

Figure 1.2 Four continuous iterative activities of fit-for-purpose biomarker assay validation.

2012) and Global CRO Council for Bioanalysis (GCC) (Hougton et al., 2012).

In general, as drug development proceeds through the typical phases, the level of validation needed increases accordingly. There is no mention of biomarkers in the EMA bioanalytical assay validation guideline. FDA's recent inclusion of biomarkers in the 2013 draft bioanalytical method validation guidance indicates that biomarkers are used for safety, efficacy, and patient selection and treatment; therefore, the data for these compounds are as important as PK data for a new drug. Method validation should be fit-for-purpose based on the objectives of the study. The level of risk for pre-Phase I studies is different from the level of risk during Phase II; therefore, method validation requirements should be modified accordingly. When deciding how much validation is required, the analyte development platform should be considered, as well as the purpose of the assay.

1.6 Challenges of Biomarker Quantitation

Biomarker quantitation can be quite challenging. One main reason is that most biomarkers are dealing with detecting diseases or toxicities in humans and animals with very low concentration level of proteins or metabolites among thousands of other proteins and metabolites. A blank biological matrix contains the analytes of interest, which makes it difficult to find clean matrices. Circadian rhythm, food intake, and emotional state may affect the biomarker level and data interpretation (Jian et al., 2012). Specificity of the assay needs to be carefully confirmed since the in vivo system tends to generate multiple isomers which may interfere with quantitation. Sensitivity of detecting a very low level of biomarkers can be also problematic. Selection of assay platforms can definitely have impact on the biomarker validity. For example, when ApoA1 biomarkers were measured using LC–MS and ELISA for the same set of samples, LC–MS data indicated there was a significant difference between smokers and nonsmokers while the ELISA failed to reveal this difference (Wang et al., 2015).

Stability of biomarkers during sample collection, storage, and analysis has significant impact on the data quality and needs to be established. A lack of consistency in sample collection and storage can invalidate a study before any data can be collected. Figure 1.3 demonstrates an example that pseudostability of biomarker fatty acid amides in blood is observed due to two opposite forces: release of fatty acid amides from red blood cells and their degradation in the blood. It was only uncovered by carefully investigating the results obtained from incurred samples and from fortified quality control samples. Otherwise, a misleading sample collection procedure would be used (Jian et al., 2010).

Many biomarkers tend to stick to the surface of containers during collection, storage, and sample processing, and this nonspecific adsorption loss needs to

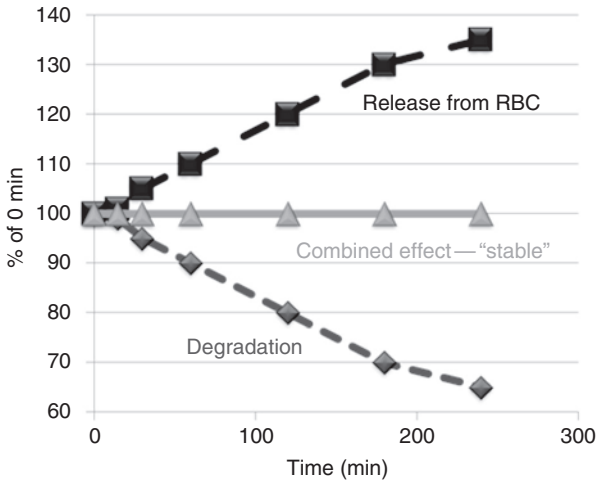

Figure 1.3 Pseudostability of biomarker fatty acid amides in blood due to two opposite forces: release of fatty acid amides from red blood cells (RBC) and degradation in the blood.

be carefully examined, resolved, or mitigated. Since there is no true blank matrix, strategy of construction of calibration standards and quality control samples should also be carefully formulated, using surrogate versus authentic matrix or surrogate versus authentic analyte (Ongay et al., 2014). A surrogate matrix is prepared using artificially prepared buffer solution containing usually a small amount of proteins to mimic the authentic matrix without the presence of the biomarker. A surrogate analyte is a stable-isotope labeled analyte that can be used to construct the analytical calibration curve even below the endogenous level of the biomarkers, thus making it feasible to quantify low level endogenous biomarkers. In order to compensate for the assay variability caused by incomplete extraction recovery and ionization suppression/enhancement from matrix components, internal standard is usually used, which is fortified to the sample in a quantitative manner at the earliest step of sample processing. The use of stable isotope labeled internal standard, which has almost identical physical and chemical properties as the biomarker analytes, provides the highest analytical specificity possible for quantitative biomarker determinations. The use of appropriate protein standards in LC–MS assays is critically important and is an active area of research within the field of protein biomarkers (Ciccimaro and Blair, 2010).

In a bioanalytical laboratory, the time used for developing a robust bioanalytical method for biomarker measurement is typically two to threefolds higher than the time used for establishing bioanalytical assay for a drug candidate.

1.7 Current Technologies for Biomarker Quantitation

1.7.1 LC–MS

Analysis of biomarkers by LC–MS has seen rapid increase in the last decade. Current advances of chromatographic stationary phases and applications of LC–MS for biomarker research were reviewed in literature (Cummings et al., 2009; Denoroy et al., 2013; Chappell et al., 2014). The advantages and applications of LC–MS for biomarker analysis are well covered in the following chapters of this book. Small molecule biomarkers can be usually analyzed directly without derivatization or with derivatization to enhance their detectability (e.g., 4-β hydroxylcholesterol) (Barnaby et al., 2015; Niwa et al., 2015; Zhu et al., 2015). Aided by the highly sensitive mass spectrometry instrument, more efficient LC, and better understanding of sample preparation, it is not unusual to achieve sub ng/mL sensitivity of measurement for many types of small molecule biomarkers (Houghton et al., 2009).

On the large molecule side, significant progress is also being made to establish robust bioanalytical assays for measuring biomarkers (Berna et al., 2008; Ackermann, 2009; Palandra et al., 2013; Wang et al., 2014). However, even with rapid progress on innovative sample clean-up procedure such as immunoaffinity capture, nano- and microflow LC for more efficient ionization, and various enzymatic digestion procedures to generate surrogate peptides, current LC–MS still lags behind on sensitivity for the measurement of many protein biomarkers by ELISA and in particular RNA/DNA biomarkers by qPCR, especially on the intact level. In the foreseeable future, it can be anticipated that LC–MS will play a complementary role for the ELISA of protein biomarkers and qPCR of RNA/DNA biomarkers. It is important to note that using different analytical methods, different conclusions may be drawn, as already discussed previously for ApoA1, a potential endogenous biomarker for cardiovascular diseases. When ApoA1 was measured by LC–MS, there was a statistically significant difference between smokers and nonsmokers while the ELISA assay for the same set of samples did not indicate that (Wang et al., 2015).

1.7.2 GC–MS

Gas chromatography in conjunction with MS (GC–MS), which predated LC–MS, offers some unique advantages for measuring small molecule biomarkers. GC typically provides higher resolution power than LC. Excellent resolution of the biomarker of interest from its interference peaks was achieved with an extremely sharp peak (Zimmermann and Jackson, 2010). A metabolomic

study of biomarkers associated with dimethylnitrosamine (DMN)-induced hepatic fibrosis in Sprague–Dawley rats was performed using GC–MS (Ju et al., 2013). This high chromatographic resolution power can come handy when chromatographic resolution of isobaric isomers of a biomarker is needed. GC–MS can also be more sensitive than LC–MS for some biomarkers. Although most biomarkers would need a derivatization step to make them volatile and suitable for GC–MS analysis, some volatile biomarkers can be analyzed directly without derivatization.

1.7.3 Ligand-Binding Assay

A typical LBA utilizes an analyte-specific binder (typically antibody but may include other binders such as binding protein, drug, target protein, or receptor) to capture the analyte of interest. The captured analyte is detected by the "detector molecule" which is generally an antibody labeled with a radioisotope (e.g., ^{125}I), an enzyme (e.g., horseradish peroxidase, alkaline phosphatase), or another label (e.g., biotin, avidin). ELISA, the most commonly used LBA, generally uses a detector molecule that is labeled with an enzyme. The extent of enzyme activity is measured by the changes in color (or fluorescence) intensity of the substrate solution. The color intensity is directly proportional to the concentration of analyte captured on the microtiter plate.

LBA is extremely sensitive and is currently still the method of choice for large molecule biomarkers such as proteins (Sloan et al., 2012). LBA also has higher throughput than LC–MS analysis. On the other hand, developing a suitable antibody can be tedious and careful control of assay parameters such as critical reagents and parallelism is very important (Stevenson, 2012; Stevenson and Purushothama, 2014). Due to the nature of indirect measurements in LBA, the results are somewhat less precise than chromatographic assays. Due to the limited analyte-binding capacity of the binder molecule (e.g., capture antibody), the typical calibration curves in these assays are nonlinear, as opposed to the linear curves in chromatographic assays. Consequently, the range of quantification in an LBA is narrower than in the linear curves of chromatographic assays. The resulting high concentration of biomarkers in the study samples may require sample dilutions. The other potential drawback of LBA is potential lack of selectivity due to cross-reactivity of the capturing antibodies with multiple compounds in the matrix.

1.7.4 Flow Cytometry

Microparticle-based flow cytometric assays for determination of biomarkers has gained tractions over the last decade (Wu et al., 2010). A large number of analytes can be measured on these multiplex systems simultaneously (Vignali, 2000). The technology utilizes microspheres as the solid support for a conventional immunoassay, affinity assay, or DNA hybridization assay which are subsequently analyzed on a flow cytometer, although the initial setup can be time consuming and expensive.

1.7.5 Quantitative PCR (qPCR)

qPCR is a powerful and sensitive gene analysis technique and it measures PCR amplification as it occurs. Typically, a qPCR program consists of a series of 20 to 40 repeated temperature changes, called cycles, with each cycle commonly consisting of two to three discrete temperature steps, usually three (e.g., 94–96°C for denaturation, ~68°C for annealing, and 72°C for elongation). qPCR is extremely sensitive (sub pg/mL range) and has become the gold standard for measuring DNA and RNA including both drugs and biomarkers in biological fluids (Wang et al., 2013a, 2013b; Wang and Ji, 2016). A reverse transcript step is needed to convert RNA into a complementary DNA template, which is then amplified with real-time detection of florescence. During amplification, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of the amplification process. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. However, it suffers from low specificity and low accuracy/precision (up to ±50%) as well as high reagent costs.

1.8 Current Biomarker Quantitation Applications

Many applications of biomarker quantitation have been reported in literature. It is not possible to have a comprehensive review in this chapter. In the following chapters of this book, a more detailed discussion of various types of biomarkers is provided. Some representative examples using various technologies discussed in the previous section are illustrated here.

1.8.1 Protein Biomarkers

Proteins are very diverse and therefore potentially informative as biomarkers. Challenges for developing new protein-based biomarkers include the complexity of protein composition in blood, the diversity of post-translational modifications, the low relative abundance of many proteins of interest, the sequence variations among different clinically relevant species, and most

importantly, the difficulties in developing suitable high sensitivity bioanalytical assays. Discovery and development of new protein-based biomarkers with proper characteristics is an expensive and time-consuming task. ELISA has been traditionally employed for protein biomarker measurement (DeSilva et al., 2003; Lee, 2009; Valentina et al., 2011). ELISA assay is very sensitive but may suffer from cross-reactivity of similar endogenous proteins.

Acute kidney injury (AKI) has been defined as a rapid decline in glomerular filtration rate. Diagnosis of AKI is frequently based on measurements of blood urea nitrogen (BUN). BUN and serum creatinine, another commonly used biomarker for AKI, are not very specific or sensitive for the diagnosis of AKI because they are affected by many renal and nonrenal factors that are independent of kidney injury or kidney function. Urinary kidney injury molecule (KIM-1) is proposed as a sensitive quantitative biomarker for early detection of kidney tubular injury (Han et al., 2002; Mussap et al., 2014). A validated sandwich ELISA assay for measuring KIM-1 in urine was reported (Chaturvedi et al., 2009). Linearity, intra-run precision, inter-run precision, lower limit of quantification, recovery, dilution verification, reference range, stability, and length of run were established. The low limit of quantitation (LLOQ) is 59 pg/mL.

CHI3L1, also known as YKL-40, a member of family 18 glycosyl hydrolases, is secreted by cancer cells. YKL-40 was determined by ELISA in plasma samples from 73 patients with relapse of ovarian cancer shortly before start of second-line chemotherapy. Plasma YKL-40 was increased in ovarian cancer patients (median 94 μg/L, range 20–1970 μg/L) compared with age-matched controls (33 μg/L, range 20–130 μg/L) ($p < 0.001$). High plasma YKL-40 is related to short survival in patients with recurrent ovarian cancer (Dehn et al., 2003). Plasma YKL-40 was also identified as an obesity-independent marker of type 2 diabetes related to fasting plasma glucose and plasma IL-6 levels (Nielsen et al., 2008).

Protein biomarkers can also be measured by LC–MS either in intact protein form (top-down) or by unique surrogate peptide generated after enzymatic digestion (bottom-up) (Liebler and Zimmerman, 2013; Percy et al., 2014). The enzyme digestion condition and selection of appropriate internal standards can have significant impact on the assay quality (Bronsema et al., 2013). The top-down approach usually uses high-resolution MS such as time of flight (TOF) MS while the bottom-up approach uses traditional multiple reaction mode (MRM). Oftentimes, immunoaffinity extraction using antibody of the target analyte is used to improve the assay selectivity and sensitivity (Carr and Anderson, 2008; Wang et al., 2012). Further selectivity/sensitivity enhancement can be achieved using dual immunoaffinity capturing procedure as in the example of quantifying interleukin 21 (IL-21) (Palandra et al., 2013). An immunoaffinity LC–MS assay for quantification IL-21 in human and cynomolgus monkey serum was developed. The workflow includes offline enrichment of IL-21 using an anti-IL-21 capture antibody, followed by trypsin digestion, online enrichment of IL-21 derived tryptic peptides using antipeptide antibodies, and quantification using nanoflow LC–MS.

Apolipoproteins are high abundance serum proteins situated on the surface of lipoprotein particles that transport highly hydrophobic lipids. Current evidence suggests that ApoA-1 is a potential diagnostic biomarker for coronary heart disease risk (Rader and Hovingh, 2014). Furthermore, the risk of coronary heart disease is strongly associated with increased adiposity, which can be further increased by smoking behavior (Slagter et al., 2013). A stable isotope dilution LC–MS method for serum ApoA-1 was developed and validated. Full validation was performed by employing nine tryptic peptides generated from native ApoA-1 in order to maximize coverage of the endogenous ApoA-1 protein. Recombinant ApoA-1 internal standard was prepared by stable isotope labeling with amino acids in cell culture (SILAC) by using [$^{13}C_6$ $^{15}N_2$]-lysine and [$^{13}C_9$ $^{15}N_1$]-tyrosine (Wang et al., 2015).

Apolipoprotein C3 (ApoC3) is one of many plasma glycoproteins which have been extensively studied for potential utility as disease biomarkers. ApoC3 is a 79-amino acid protein synthesized by liver and intestine. ApoC-3 has a critical role in the metabolism of triglyceride (TG)-rich lipoproteins (TRLs) (Norata et al., 2015). Previously, an LC–MS assay using a solid-phase extraction (SPE) method for the plasma sample preparation was published. This "top-down" approach provided intact analysis of ApoC3 glycoisoforms and potential for data mining, and high-resolution MS afforded excellent specificity. The assay was also applied to analysis of plasma samples collected from normal, prediabetic, and diabetic subjects for preliminary evaluation of the biomarker potential of ApoC3 glycoisoforms for early diagnosis of diabetes. The results showed that there was a significant difference among the different groups (Jian et al., 2013).

1.8.2 Peptide Biomarkers

Peptides can be an important class of biomarkers. Traditionally, peptide biomarkers in biological samples have mostly been analyzed by immunoassay methods. Similar to protein biomarkers, cross-reactivity with structurally related peptides prevents selectivity. The combination of a separation technique such as micro/nano-HPLC with a detection method as MS is a very selective and sensitive approach and permits the

simultaneous analysis of a great number of peptides (Saz and Marina, 2008).

The 40- and 42-amino acid residue forms of β-amyloid (Aβ1–40 and Aβ1–42) in CSF have been proposed as potential biomarkers of AD (Whiley and Legido-Quigley, 2011). In 2006, an immunoaffinity purification and LC–MS assay was developed for analysis of amyloids in CSF (Oe et al., 2006). In another report, a mixed-mode SPE method and an ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) assay was developed for the simultaneous quantitation of Aβ1–38, Aβ1–40, and Aβ1–42 from human CSF (Lame et al., 2011). Analysis of Aβ peptides in plasma has its own methodological challenges, including binding to plasma proteins and carryover of analytes from previous injections when using LC (Goda and Kobayashi, 2012).

There is also a substantial evidence that β-amyloid peptide is oxidized in vivo, which has led to the suggestion that oxidative stress might be an important mediator of AD. Trypsin digestion of both native and oxidized Aβ1–16 and Aβ1–40 resulted in the formation of tryptic peptides corresponding to native and oxidized Aβ6–16, which could be separated by LC. Sites of oxidation were then unequivocally characterized as histidine-13 and histidine-14 by LC–MS analysis of the tryptic peptides (Inoue et al., 2006).

1.8.3 RNA Biomarkers

Micro RNAs (miRNAs) are small noncoding RNAs found in eukaryotic organisms that regulate gene expression. Dismissed as "junk" until about a decade ago, it is now widely accepted that they play an important functional role in a wide array of cellular processes. miRNAs play important regulatory roles in many cellular processes, including differentiation, neoplastic transformation, and cell replication and regeneration. Many studies have demonstrated that dysregulation of these miRNAs is associated with various diseases suggesting there is potential for use of miRNAs in diagnosis and treatment. Arguably, secreted miRNAs have many requisite features of good biomarkers. They are stable in various bodily fluids, the sequences of most miRNAs are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be easily assessed by various methods, including methods such as PCR, which allows for signal amplification. Much of the study of miRNA and disease has focused on cancer and neurological disorders (Ju, 2010). A number of bioanalytical challenges exist for the analysis of miRNAs in biological fluids as very nicely summarized by Wang and Ji (2016) and by Basiri and Bartlett (2014). While the PCR and hybridization ELISA give the best sensitivity, LC–MS shows promise to be the next generation analyzer for this type of molecules due to its sensitivity for small oligonucleotides (<25-mer), broader dynamic range (up to 3 orders of magnitude), no need for specific reagent, and the capability to quantify intact double-stranded oligonucleotides (siRNA) and their metabolites.

1.8.4 Nucleotide Biomarkers

Plasma concentrations of nucleotides such as AMP, ADP, and ATP provide information on their relative physiological importance in regulatory mechanisms and therefore could be useful biomarkers. Analytical approaches of determining AMP, ADP, and ATP in biological samples have been proven challenging due to their high polar nature. Zhang et al. discussed a novel, fast, highly sensitive, selective, and validated ion-pairing hydrophilic interaction chromatography (HILIC)–MS method utilizing diethylamine (DEA) and hexafluoro-2-isopropanol (HFIP) in the mobile phase and an aminopropyl chromatographic column (Zhang et al., 2014).

1.8.5 Small Molecule Biomarkers

One of the great promises of the metabolomics approach is the fact that groups of metabolite biomarkers are expected to be less species-dependent than gene or protein markers, facilitating the direct comparison of animal models with human studies, which in turn improves the potential of the technique to rapidly convert laboratory-based research into clinical practice (Barr et al., 2010). Typically, LC–MS-based technologies are used for small molecule biomarker analysis. Challenges of analyzing small molecule biomarkers include separation of isobaric position isomers which have identical molecule weight to the analyte of interest, poor retention due to extremely polar nature, poor sensitivity due to lack of favorable ionization function groups, and poor stability. Derivatizaion strategy has been frequently used to enhance sensitivity and selectivity (Meyer et al., 2011). Novel chromatographic stationary phase such as HILIC can be used to enhance the retention and thus the sensitivity of polar biomarkers (Weng, 2001; Jian et al., 2011; Li et al., 2012). Care should also be exercised to prevent introducing artifacts during the sample storage and processing (Chao et al., 2008).

Cytochromes P450 3A4 (CYP3A4) and CYP3A5 are important human drug metabolizing enzymes with high interindividual variability in hepatic and intestinal activities. DDI with CYP3A4-inhibiting drug such as itraconazole or inducing drug such as rifampin can dramatically change the CYP3A4 activity in man. Therefore, regulatory agencies such as FDA and EMA have issued guidelines on assessing DDI mediated by P450 enzymes

including CYP3A4, 2D6, 2C9, 2C19, and so forth, and various transporters. The most widely used and accepted method to assess CYP3A activity is to examine midazolam PK. Urinary 6β-hydroxycortisol to plasma cortisol metabolic ratio has also been used historically as a noninvasive measure of CYP3A activity (Lutz et al., 2010), which is a more rapid biomarker due to short half-life with little delay time behind the changes of CYP3A4 activity in vivo. However, diurnal effect leads to more variable data. Plasma 4β-hydroxycholesterol is an endogenous metabolite of CYP3A4-mediated cholesterol metabolism and has been extensively investigated. It is the first choice if a stable biomarker is needed. The long half-life of 4β-hydroxycholesterol results in small variations in its concentrations but excludes this marker in short-term studies. Using both biomarkers in clinical studies would be recommended if the outcome is unknown (Mårde Arrhén et al., 2013; Dutreix et al., 2014).

24S-hydroxycholesterol (24S-HC) can be formed from cholesterol via cytochrome P450 family 46A1 (CYP46A1, cholesterol 24-hydroxylase) in brain. 24S-HC is capable of passing across the blood–brain barrier and enters the systemic circulation. Therefore, the plasma concentration of 24S-HC can be used as a marker for cholesterol homeostasis in the human brain (Lutjohann et al., 1996). Sugimoto et al. reported a highly sensitive and specific LC–MS method with an atmospheric pressure chemical ionization interface to determine 24S-hydroxycholesterol in plasma (Sugimoto et al., 2015). Phosphate-buffered saline including 1% Tween 80 was used as the surrogate matrix for preparation of calibration curves and quality control samples. The saponification process to convert esterified 24S-hydroxycholesterol to free sterols was optimized, followed by liquid–liquid extraction using hexane. Chromatographic separation of 24S-hydroxycholesterol from other isobaric endogenous oxysterols was successfully achieved with gradient elution on a C18 column. This assay was capable of determining 24S-hydroxycholesterol in human plasma (200 μL) ranging from 1 to 100 ng/mL with acceptable intra- and interday precision and accuracy.

1.9 Conclusion and Future Perspective

There is no doubt that mass spectrometry-based technologies will continue playing major roles for biomarker research including quantitation, especially for small molecule biomarkers and peptide biomarkers which arguably provide more direct links to a biological process in vivo since many of these small molecules or peptides are the direct substrates of these biological processes. There are also many mature technologies available and wealthy application information from literature. Seldom did we fail to develop an assay to quantify this type of biomarkers even though some of them can be quite challenging. We will continue see the use of fit-for-purpose approach in the assay establishment so that the right resources and costs are utilized at different stages of drug discovery and development programs. Continual dialog between industry and regulatory authorities will lead to better and more practical solutions on biomarker quantitation.

Challenges are still ahead of us. Both protein and miRNA biomarkers present significant challenges for LC–MS bioanalysis. Proteins can be measured by LBAs but they suffer potential cross-reactivity with similar proteins which may exist in much high quantity in the samples. Current bottom-up approach (use of surrogate peptide after enzymatic digestion to reflect the intact protein biomarker), while more sensitive than the top-down approach, requires extensive method development and thorough understanding of structure modifications of protein in the body. The top-down approach which utilizes the high-resolution MS detection is less subject to quantitation bias due to protein modifications but is significantly less sensitive and is currently only limited to abundant protein biomarkers. miRNA can be measured by qPCR but the procedure is tedious and assay accuracy is less than desirable to support biomarker utilization with small to moderate changes. Attempt of using LC–MS for miRNA biomarkers is made but all these LC–MS assays suffered from poor sensitivity due to unfavorable ionization of RNA type of molecules, compounded by use of ion-pair reagents or high level of buffers in the mobile phases, typically for RNA/DNA molecules in order to maintain good peak shape.

We will continue to see the improvement of sensitivity by using sample preparation technologies such as immunoaffinity extraction which not only allows cleaner extraction but also provides analyte enrichment. Currently, it is quite costly to use such an approach. Hopefully with more commercialization of antibodies and more automation, the cost will come down significantly. We will also see the use of more applications and refinements of using nano- or micro-LC for the sensitivity enhancement. The lack of system robustness and ability of swift switch of assay parameters are the current limitations, especially for the nano-LC system, which prevents the full utilization of such systems in support of discovery programs where the same instrument needs to support multiple programs with much diversified chemical structures. We will also see some of the enhancement of additional separation capabilities such as ion-mobility device that can assist in separating isomers in the ionization sources. Nevertheless, the Achilles heel of LC–MS-based technologies is the inadequate

sensitivity to measure low abundant protein or RNA biomarkers. In order for LC–MS to be more universally applicable to quantifying protein and RNA biomarkers, the absolute sensitivity of mass spectrometer, especially high-resolution mass spectrometer, must be significantly improved.

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