20 The Role of Biomarkers in Drug Discovery and Development: Enabling PK/PD

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20.1 SUMMARY

The ultimate goal of drug discovery and development is to find the right drug and to administer it at the right dose with the right frequency to the right patient. Biomarkers can be an invaluable tool to meet this goal by confirming the efficacy and safety of drug candidates, enabling the understanding of their mechanism of action (MOA), and determining the efficacious dose and dosing regimen by establishing the correlation between the pharmacokinetics (PK) and pharmacodynamics (PD) of the drugs. They can also aid the selection of a patient population that will respond to treatment and benefit the most from their therapeutic effects, thus creating the path for personalized medicine. Biomarkers are objectively measured indicators of pathogenic processes of disease development or responses to therapeutic intervention. This chapter provides a brief description of the key concepts related to the use of biomarkers in pharmaceutical drug discovery and development. The role of target, mechanism, outcome,

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and surrogate biomarkers in the early stages of drug discovery, starting with target identification, nonclinical model development, through clinical translation and proof of concept (POC) applications are described. A brief section on PK/PD-model-based drug development and translational research illustrates how technological advances enabling more accurate and robust assessment of drug concentrations in target tissues and systemic circulation together with PD response data lead to more accurate human dose projections and more optimal clinical trial designs. Considerations for the characteristics of optimal PK/PD enabling biomarkers are presented, including study design and sampling considerations and an overview of the most commonly used methodologies for the development of quantitative biomarker assays. Since assay development and validation for biomarkers can take significant time and is expensive, the importance of biomarker validation using fit-for-purpose assays is discussed. The challenges and solutions to various aspects of the fit-for-purpose validation are described in the context of the drug development stage requirements. Throughout the chapter, historical perspectives and representative examples for the challenges, solutions, and applications of biomarkers are provided.

20.2 INTRODUCTION

Biomarkers have been used for over 100 years in medical practice and have been playing a key role in drug discovery and development for over half a century. The National Institute of Health Biomarker Definitions Working Group defined biomarkers as various biochemical, physiological, imaging, and behavioral characteristics that are objectively measured as indicators of normal or pathologic processes or in response to therapeutic intervention [1]. Perhaps, the two most well-known examples of biomarkers are blood glucose for insulin-dependent diabetes and low density lipoprotein cholesterol (LDL-C) for hypercholesteremia and cardiovascular disease. Their history illustrates that the discovery, validation, and application of biomarkers in medical therapy and drug discovery require interdisciplinary research between biology, medicine, and drug development and can take several years to decades. The earliest records of diabetes can be found in Egyptian papyrus records in 1552 BC, mentioning frequent urination as the symptom, and from the eleventh century, diabetes was diagnosed by tasting the urine of subjects as its sweet taste was connected to the disease. However, it was not until the nineteenth century that the first chemical tests to measure sugar in the urine were developed. Furthermore, it took over 100 years of medical and biology research to discover and link insulin with type 1 diabetes, with the first successful treatment of a depancreatized dog with insulin occurring in 1921. The first home tests for urinary glucose became available in the 1960s, and Clemens' patented blood glucose meter in 1971, enabled the easier monitoring and medication management of diabetics [2]. Closer monitoring of the glucose biomarker levels combined with adaptive therapy, applying more frequent doses and self-adjustments according to individual activity and eating patterns, have significantly delayed the onset and progression of long-term complications in diabetic patients, thus illustrating the importance and utility of the biomarker in managing clinical outcomes for diabetes [3]. As for the history of LDL-C, the concept of using biomarkers in the prevention and treatment of cardiovascular diseases can be traced back to the Framingham study [4]. The investigators initiated this study in 1949 to "seek a single essential cause to produce cardiovascular disease."

It was soon realized that complex and multifactorial interactions lead to the pathogenesis of atherosclerotic cardiovascular disease. However, it was through this study that the quantitatively measured clinical parameters of total cholesterol and LDL-C as traditional risk factors for coronary heart disease were identified. Subsequent pharmaceutical research to identify drugs that inhibited cholesterol synthesis led to the discovery of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that we now know as "statins." Statins were approved in 1987 to lower total cholesterol and LDL-C, and in 1994, statins were shown to reduce cardiovascular events [5].

As illustrated by the above examples, there are a number of important roles that biomarkers play in clinical applications including diagnosis, monitoring disease progression or reversal, and patient selection for clinical trial stratification. In recent years, modern drug discovery has recognized the importance of biomarkers in the earlier stages of drug discovery and development. Their utilization is often required in compound selection strategies for nonclinical development using the efficacy and/or safety biomarker profile of drug candidates, as well as for the proof of medical hypothesis by linking drug effect to the biological target using relevant and validated target and mechanism biomarkers [6].

20.3 DEFINITION OF BIOMARKERS AND THE ROLES THEY PLAY IN DRUG DISCOVERY AND DEVELOPMENT

20.3.1 Target, Mechanism, Outcome, and Surrogate Biomarkers for Drug Discovery and Development

The drug discovery and development process consists of several stages. It starts with the identification of viable targets and continues with the screening and selection of potent drug candidates against the target. This is followed by testing these potential candidates to confirm their nonclinical *in vivo* efficacy and safety prior to proving their safety and efficacy in humans and ultimate benefit to the patients. In the candidate screening and selection process, it is important to demonstrate if a drug candidate is truly interacting with the target by directly or indirectly preventing it from performing its biological function, and if this interaction will result in modifying the disease. *Target biomarkers* provide feedback on direct inhibition of the target enzyme. *Mechanism biomarkers* provide information on the target inhibition, downstream from the target, in the disease pathway. *Outcome biomarkers* are indicative of the clinical outcome of the disease and are sometimes referred to as *surrogate biomarkers* since they are indicative of how the patient feels and functions. These concepts and definitions are illustrated by the examination of the well-known cholesterol synthesis pathway shown in Fig. 20.1. The first step in this pathway is the conversion of acetyl-coenzyme A to mevalonic acid (MVA) by HMG-CoA reductase. The target enzyme is HMG-CoA with the hypothesis that its inhibition will result in reducing blood cholesterol. MVA is the product of the first step in the enzymatic synthesis and thus is the target biomarker for this pathway. The other intermediates in this pathway are potential mechanistic biomarkers, while LDL-C is the outcome biomarker. On the basis of the results of the Framingham study, LDL-C has been validated as an outcome biomarker for cardiovascular disease. Since the connection between coronary heart disease and LDL-C is scientifically and medically well established, it is also accepted as a validated surrogate biomarker for

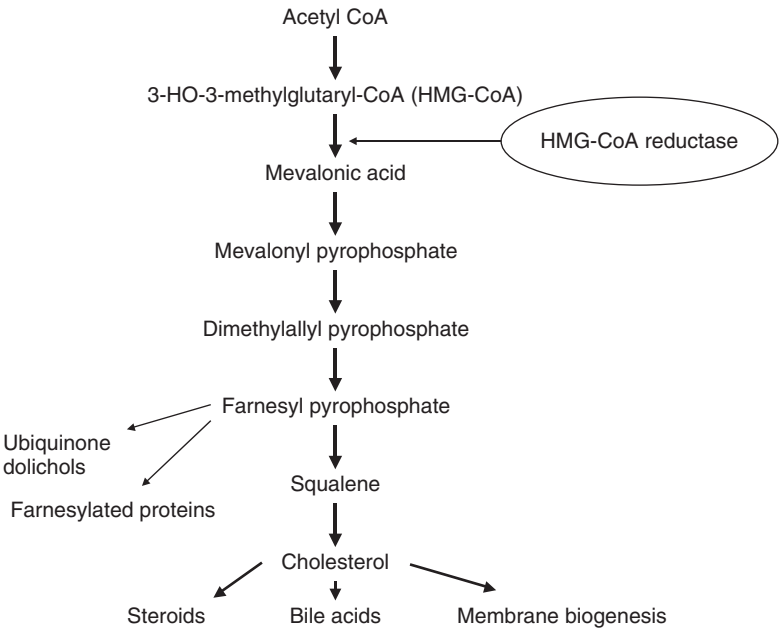


Figure 20.1 Schematic of the cholesterol biosynthetic pathway. Mevalonic acid is the target biomarker for the conversion of HMG-CoA by HMG-CoA reductase [19].

this disease. Validated target, mechanism, and outcome biomarkers are invaluable in drug discovery and development as they can lead to earlier validation or disqualification of a new target, provide feedback on drug design and selection, and save significant time and cost. For instance, by demonstrating that a drug candidate "hits" the target via inhibiting the formation of the target biomarker, but results in no modulation of the outcome biomarker that has been linked to the disease state, a target can be proved nonviable. Similarly, if target modulation was not achieved *in vivo* then this information can be provided to the medicinal chemist to design and synthesize more potent candidates. Another benefit is time and cost savings. For instance, confirming modulation of cholesterol synthesis by measuring the steady state concentration of systemic LDL-C can take four to six weeks, while potent inhibitors of HMG-CoA show modulation of the target biomarker, MVA, less than 24 h post dose in the blood of patients, thus enabling the evaluation of potential novel compounds much faster and at lower cost. As illustrated in Fig. 20.2, acute changes in plasma mevalonate levels have been demonstrated to correspond to the potency of statins. In addition, since the target organ for statins is the liver, plasma drug concentrations can be difficult to measure, so the plasma biomarker modulation facilitates the assessment of target inhibition. Therefore, plasma mevalonate can be used in succinct nonclinical PK/PD modeling studies to select an efficacious dose and is translatable from nonclinical models to humans.

20.3.2 Disease Biomarkers and Diagnostic Markers

As described above, outcome or surrogate biomarkers that are well understood and validated for a given disease state can be utilized to diagnose or confirm a disease. It is

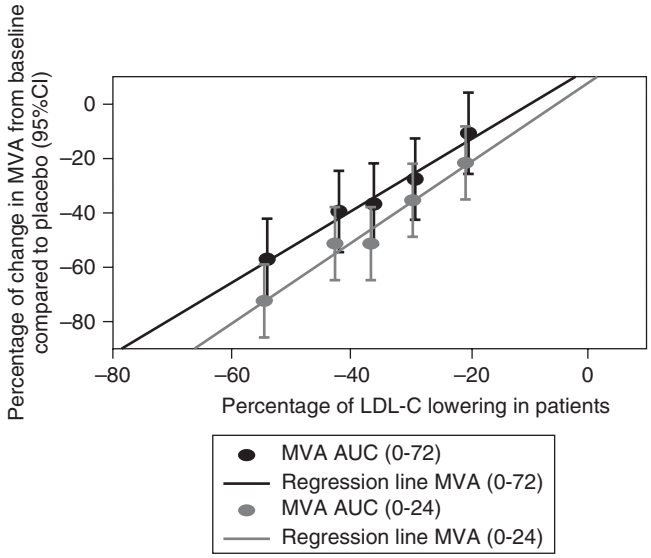


Figure 20.2 A relationship exists between the change in mevalonic acid (MVA) area under the curve (AUC) following a single dose of statins in healthy subjects and steady state changes in LDL-C in patients.

important to note that the normal range of some biomarkers can have significant inter-subject variability. Therefore, it is not the actual biomarker levels, but changes to these levels, that are diagnostic of a developing or advancing disease state. For instance, until recently, research on one of the most widely used tumor biomarkers, prostate-specific antigen (PSA) for prostate cancer, suggested that the rate of increase in PSA (the PSA velocity) was a more specific marker for prostate cancer than the absolute blood levels [7]. Thus, it was recommended that baseline levels were established for subjects in the higher risk populations and monitored for changes with a given frequency (i.e., approximately two years). The rate of PSA increase is also believed to have a value in prostate cancer prognosis. For instance, men with prostate cancer whose PSA level increased by more than 2 ng/mL in a year before the diagnosis have a higher risk of death from prostate cancer despite surgery [8]. Most recent PSA diagnostic tests approved by the Food and Drug Administration (FDA) between 2000 and 2010 are actually used to determine the ratio of free PSA to that of total PSA, including the fraction that is bound to serum proteins. In men with prostate cancer, the ratio of free to total PSA is decreased, and the risk of men acquiring cancer increases if the free to total PSA ratio is less than 0.19–0.25 [9].

20.3.3 Biomarkers for Patient Selection and Trial Stratification

A great promise of modern drug development is personalized medicine that enables the physician to select the treatment most beneficial to the patients to cure, or slow down the disease with the least amount of risks from unwanted side effects. Biomarkers, often codeveloped and validated as a diagnostic, with a given therapeutic can enable the selection of the patients who are most likely to respond to the treatment, thus obtaining

the most benefit. The development of companion diagnostics became possible because of the increased understanding of the role that particular genes play in the disease pathology.

Examples of this for cancer are Herceptin® that is approved for the treatment of human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer tumors, as well as Vectibix® and Erbitux® that are approved for V-Ki-ras2 Kirsten Rat Sarcoma viral oncogene homolog (KRAS) expressing colorectal cancer. The cytochrome P450 genotyping test approved in 2003 by the FDA using gene chip arrays can be used to determine if a patient has mutations in their CYP4502D6 gene that can affect their ability to metabolize certain drugs, which can lead to harmful drug–drug interactions or inappropriate dosing and treatment. Human gene expression analysis already plays and will continue to play an increasing role in the identification and validation of new disease targets and the development of individualized medicine with more effective and safer treatment options [10–12].

20.3.4 Pharmacodynamic Biomarkers

Biomarkers are also a means to assess PD response. Their use in understanding effect and exposure relationships is essential for PK/PD-model-based drug development enabling better predictions of efficacious dose and regimen [13–16]. Target and mechanism biomarkers that are directly linked to a target's activity or its MOA through a given biochemical pathway or outcome can potentially be used as PD markers. Their full utilization requires translatability between nonclinical models and human, validation of the animal models for nonclinical *in vivo* efficacy screening, as well as confirming the MOA in POC clinical studies. Blood concentrations of drug as a result of therapeutic dosing have long been assessed to determine PK parameters; thus, they can be considered a biomarker of dose response. On the other hand, in some cases, the blood concentrations of drug cannot be measured because of exposure levels below the limits of detection of the technology used or rapid conversion of the parent drug to metabolites. In these instances, PD biomarkers are often recommended to help to establish tolerated doses and dosing regimen.

20.4 PHARMACOKINETIC/PHARMACODYNAMIC-MODEL-BASED DRUG DISCOVERY AND DEVELOPMENT

20.4.1 PK/PD-Model-Based Drug Discovery and Development

The goal of PK/PD modeling is to link the drug exposure to the pharmacologic response and thus determine the optimal dose and dosing regimen to attain and maintain an efficacious concentration for successful treatment. In recent years, significant advances have been made in this area due to the development of more robust bioanalytical methods to determine PK parameters by measuring circulating drug concentrations systemically and in target organ tissues, as well as measuring biochemical PD biomarkers. An example of this is the use of physiologically based pharmacokinetic (PBPK) models to characterize multivariate systems [13]. PBPK modeling is a mathematical modeling method that is used for predicting the absorption, distribution, metabolism, and excretion (ADME) of a compound *in vivo*. PBPK models are also usually multicompartment models in which the compartments correspond to predefined organs

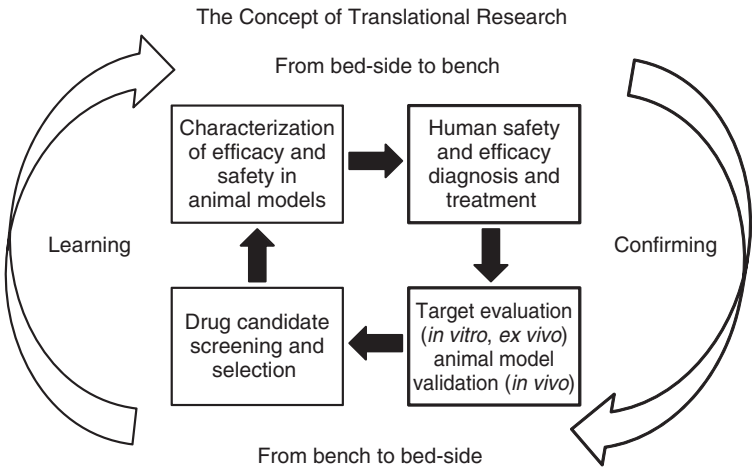


Figure 20.3 Translational research process illustrating the bidirectional and iterative integration of nonclinical basic research (learning) and its application after clinical translation (confirming).

or tissues in the body, and the interconnections correspond to blood or lymph flows. Measurement of drug concentrations systemically (in plasma or serum) and in the target organs, as well as biomarkers that correspond to the PD response in these same compartments, enables the development of more robust PBPK and PBPK/PD models resulting in better predictions of optimal dose.

20.4.2 Translational Research

Translational research is a recent interdisciplinary approach that focuses on successfully advancing fundamental discoveries from the discovery to clinical setting, and is often interpreted to include a bidirectional component where clinical findings are integrated back into the nonclinical space [17–19]. More broadly, this bidirectional component can be interpreted to include a 'learning and confirming' model of iteratively increasing and applying knowledge from nonclinical to clinical testing [20]. The basic components of this translational research process are illustrated in Fig. 20.3. Owing to the sequential process of drug discovery and development from *in vitro* and *in vivo* nonclinical models to human, it is paramount that the translatability of nonclinical knowledge to clinical relevance, and vice versa, is well understood and integrated in a successful and more efficient drug development process.

20.5 PK/PD ENABLING BIOMARKERS

As defined previously, PD is the science of drug action on an organism. It may be studied at many organizational levels (molecular, cellular, tissue/organ, and whole body) and at all stages of the drug development process. In addition, by enabling the quantitative integration of information across different species and throughout the clinical phases of development, PD measures can be represented by a number of

TABLE 20.1 Characteristics of Optimal PD Biomarkers

Biomarker Attribute	Desired Characteristic for PD Modeling
Mechanism of action	The biomarker's connection to the mechanism of action and disease state is well understood and correlated
Translatability	Biomarker is expressed in nonclinical models and clinical subjects and is correlated with the mechanism of action
Biological variability is well understood	Intra- and intersubject variability is well understood, the range of biological variability is low relative to modulation with disease state and drug effect
Accessibility in biological matrix	Sample collection is noninvasive, small sample size preferred
Measurability	Objective, quantitative techniques can be applied

different biomarker classes and utilize a wide range of laboratory and analytical techniques. Therefore, as an integral part of PK/PD modeling, the selection of effective PD biomarkers has become an essential strategy to optimize drug development resources.

20.5.1 Characteristics of Optimal PD Markers for PK/PD

When considering a PD biomarker for conducting modeling and simulation in pharmaceutical development, there are several attributes of the biomarker that would be regarded as ideal for this purpose (Table 20.1). Among these traits are its linkage to the drug/target MOA and linkage to the disease state, translatability between nonclinical models and the clinic, biological variability and modulation, and accessibility for sampling and measurement. For example, plasma and urine concentrations of MVA have been shown to correlate with the biosynthetic rate and long-term changes of cholesterol levels in nonclinical models and humans [21–24]. Therefore, determining the concentration of MVA in these accessible (peripheral) biological fluids can be used as a mechanistic biomarker of HMG-CoA reductase inhibition localized in a sequestered cellular target space (e.g., liver) and can thus provide a quantitative link between target and outcome (cholesterol level). In addition, information that has been collected on PD biomarkers that have been successfully translated to the clinic can be fed back to discovery efforts to further optimize drug candidates and nonclinical models [25]. The biomarker should also reflect the underlying biology by demonstrating a measurable impact on the biological mechanism or pathway that confirms a drug target has been hit and is having a correlating effect on the disease outcome [26]. In this regard, biomarkers that are more proximal to the drug target, rather than distal clinical end points further down the disease pathway, may be more desirable because they could minimize the influence of potential intervening variables between the target and outcome.

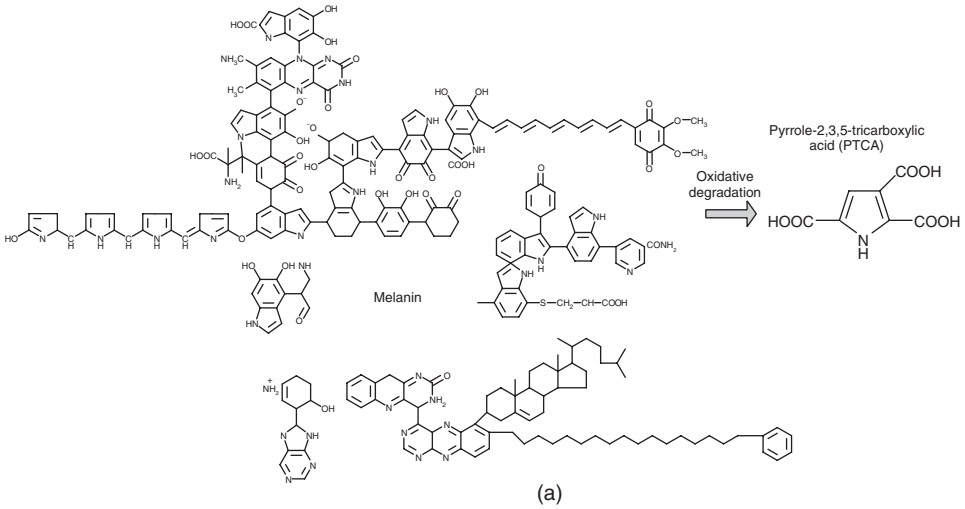
An ideal biomarker for effective PD modeling purposes should not be subject to a large amount of "noise." Biological variability, such as marked circadian and diurnal rhythms, food effects, induced stress, or various disease states, may mask changes in biochemistry that are caused by a mechanistically based drug intervention [27]. The biomarker should also have an adequate modulation window between treated and nontreated subjects, dosed groups, or healthy versus diseased, so that a change can be

quantified relative to inherent accuracy and precision errors of the employed analytical methodology. It is also beneficial if the biomarker can be quantified with objective versus subjective techniques. For example, clinical trials for pigmentary disorders involve measurement of melanin. Melanin is a highly insoluble pigment of varying composition that is not particularly amenable to commonly used quantitative methods. Therefore, instruments that measure the ratio of reflected light to incidental light from skin, such as reflectance spectrometers and tristimulus colorimeters, are increasingly being used by dermatologists for the quantitative measurement of melanin [28]. While this approach can provide noninvasive information for the melanin content of skin *in vivo*, interobserver bias or characteristics of a particular instrument's aperture limit the accuracy and reproducibility of these measurements. Therefore, a biomarker that can be developed, which reflects the skin melanin content in absolute quantitative terms, such as pyrrole-2,3,5-tricarboxylic acid (PTCA), could be a more desirable methodology. Once melanin is oxidized to its unique monomers, such as PTCA (Fig. 20.4), melanin content can then be accurately determined by validated analytical technology such as liquid chromatography tandem mass spectrometry (LC-MS/MS). These more objective measurements of melanin concentration in skin are less susceptible to interobserver or instrumental bias and can provide more reliable PK/PD modeling. Another important consideration is to understand how the concentration of a particular biomarker can be influenced by the external influences (such as diet), endogenous biosynthesis rates, and interacting biochemical pathways. Static (as a single time point) and kinetic (as multiple time point or time course) measurements can provide more insight into the turnover and synthesis rates [29]. For instance, it is well known that carbohydrate and fat intake can affect the plasma lipid profiles. Measuring the lipid concentration at a single time point (static concentration) provides a snapshot in time, but it does not reveal the origin of the lipid (endogenous vs dietary). Stable isotope tracers combined with static and dynamic (metabolomic and fluxomic) measurements can provide a more complete assessment of the biomarker modulation [29,30].

There are also practical considerations when considering an optimal PD biomarker for PK/PD. A biomarker that can be obtained in a noninvasive manner from a matrix that is easily accessible and is relatively abundant has several advantages, such as being more cost effective because of ease of sampling, the use of fewer study subjects, and greater clinical study compliance [31]. Biomarkers requiring a small sample size for their quantification also facilitate the ability to collect more time points from the same subject. This advantage is compounded if several relevant biomarkers can be multiplexed into a single quantitative analytical method. Similar to xenobiotic analysis, it is also important to assess if the biomarker has issues that could complicate sample handling, such as stability or induced changes post collection, which may interfere with accurate endogenous measurements. Finally, in a resource-constrained environment, quantitative method development for the analysis of biomarkers tends to be tool driven. Therefore, when a biomarker must be available in time for the intended use in a program, some classes of biomarkers may be prohibitively difficult to quantify accurately because of their molecular composition and the available technology.

20.5.2 Fit-for-Purpose PK/PD Markers

Developing and applying PD biomarkers for mechanistic PK/PD modeling and simulation imparts value and a quantitative basis for translational research throughout the



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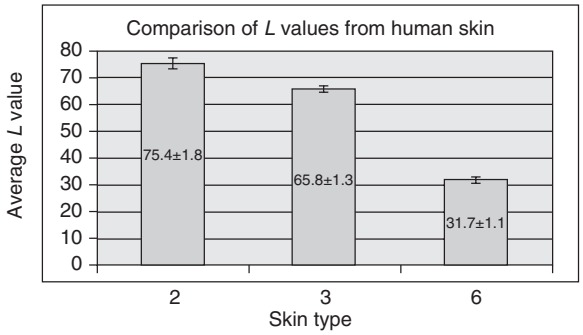

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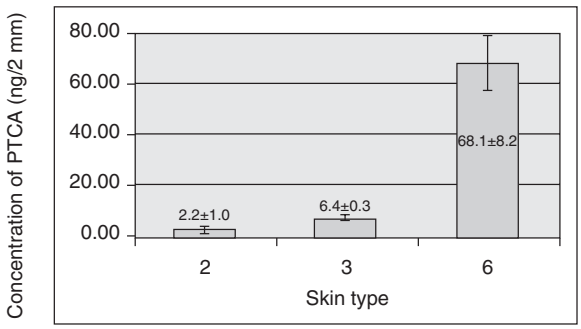

(c)

Figure 20.4 (a) Melanin can be oxidatively degraded to its unique monomers which are more amenable to common quantitative analytical methodology such as LC-MS/MS. (b) Noninvasive chromametric measurement. Melanin can be measured noninvasively using light reflecting technology to provide subjective pigment measurements, with lighter skin color reflecting more light and thus giving a larger calculated *L* value (arbitrary value obtained from chromametric measurement). (c) Comparison of PTCA concentrations obtained from different skin types. However, more objective and quantitative levels of melanin can be determined using LC-MS/MS following its conversion to PTCA monomers in 2-mm skin punch biopsies. *Source*: Part (b) and (c) graphic courtesy of Kim Wade.

drug development pipeline. The intent of this approach is to provide informed decision making in early drug development that can be applied at later stages to reduce cost through smaller study sizes or duration. Therefore, it is important to develop biomarkers, as well as the analytical tools for measuring them, that are fit for the purpose of answering the PD question that is being posed.

The desired characteristics of fit-for-purpose biomarkers depend on their intended use and the stage of drug development. For example, biomarkers selected to validate a therapeutic target or mechanism may not be appropriate for accurately scaling dose predictions across species or to form the basis of clinical trial simulations. Once the degree of confidence in mechanism has been established for candidate drugs that have an effect on this target, then the utility of the biomarker at different stages in the drug development process can be considered. If monitoring the biomarker provides a high linkage between target/mechanism and predicting dose/outcome then this could potentially be used for later definitive nonclinical or clinical PK/PD study analyses using a validated assay. For example, androgen levels within prostate tumor cells may be used as target or mechanistic biomarkers for therapies, such as Abiraterone, developed to block intracellular CYP17 enzymes and thus combat castration-resistant prostate cancer [32]. These steroid determinations may be highly useful early on in drug development to demonstrate proof of mechanism and validate the target in xenograph or *in vitro* systems. However, because of the invasiveness of sample collection, analytical challenges in their measurement, and narrow modulation window, other biomarkers such as PSA may be needed for later stage PK/PD studies and patient treatment decision making.

20.5.3 Utility of Quantitative and Qualitative Biomarker Assays

At the cellular and whole animal level, drugs can act on many biochemical pathways and in many target tissues. These actions could lead to primary responses which, in turn, may induce secondary responses, that can either enhance or diminish the primary response. Systems biology is an emerging discipline that evaluates how individual components of a complex biological system dynamically interact within the system [33]. When given a pharmacological treatment that is aimed at single components of a pathophysiological process, systems biology can provide a collective understanding of how the whole system is affected. If effectively integrated in early drug discovery and hypothesis driven, systems biology could offer an understanding of the MOA of new molecular entities, allow the modeling of different pathways individually, and estimate their overall contribution to the net effect.

Therefore, when doing studies to understand systems biology and PD hypotheses related to early validation of a disease target, more multiplexed or qualitative assays could be more appropriate than a highly accurate and precise quantitative method developed for a single biomarker. The application of "polyomic" technology, such as genomics, proteomics, lipidomics, and metabolomics, offers the potential to generate more successful drug candidates because each technology offers more extensive and complementary approaches to the underlying biological and pathological processes being examined [34]. While producing large quantities of biological data, there is already evidence that these tools can be used to detect specific changes that predict disease and drug response [35–38]. Often without reference standards, or with the goal of doing a pattern comparison between a panel of biomarkers from diseased versus healthy or treated subjects, it may be adequate for scientists to use relative quantitative

or semiquantitative assays [27]. An understanding of the basic attributes of the individual technologies, for example, sensitivity and signal-to-noise, is required to determine their applicability. For example, microarray tools that provide a comprehensive survey of the cellular transcriptional landscape and identify gene expression patterns can predict or monitor drug responses. Advances in mass spectrometry (MS) have allowed scientists to monitor protein expression changes and correlate them to disease progression. Similarly, many imaging modalities, such as magnetic resonance imaging (MRI), have been successful for qualitative biomarker analysis.

20.5.4 Study Design Considerations for Biomarkers

The biomarker strategy should be integrated with the PK/PD strategy of a program to understand confidently the relationship between dose and the time course of biomarker response. Drug exposure and biomarker response relationships *in vitro* and in nonclinical species must be understood in order to enable PK/PD modelers to evaluate the effect of time on response (e.g., modulation window, delayed biomarker responses or responses lasting longer than exposure) and will have impact on the protocol design of the studies. Therefore, the utility of the results from a PD study can only be maximized when the study design considerations for biomarkers are adequately considered.

For an ideal PD biomarker, the signal should be quantitative and reflect activity of the compound in the tissue of interest, which is relevant to the indication. The biomarker response should also be evaluated over a wide range of doses and exposures in single and/or multiple dose studies and include sampling times that provide an understanding of the relationship between time of dosing (i.e., exposure) to biomarker response. Similarly, the relationship between biomarker response and efficacy must be understood to establish the degree of biomarker signal associated with the desired outcome. This will also establish the dynamic range, or modulation window, required for the biomarker assay. In addition, whether intermittent high levels of pharmacological activity or constant levels of pharmacological activity are preferred for efficacy must be correlated to the biomarker disposition. This may come from nonclinical models, or from studies that compare the biomarker signal in normal subjects with various levels of disease activity [25]. Some direct pharmacological effects may not be rapidly reversible or may have irreversible downstream effects on the disease. For example, repeated dosing can result in toleration, sensitization, or various reflexive feedback mechanisms. Therefore, doses selected should result in clearly separated levels of pharmacological activity based on biomarker data and exposure–response modeling [38].

There are a number of other factors, both intrinsic and extrinsic, that may affect biomarker calculations that should be considered when designing PK/PD studies [27,39,40]. For example, animal species and biological sample size (i.e., animal size) or availability may limit the amount of biological sample that may be collected from each subject and may increase the number of subjects needed for each study or necessitate the use of highly sensitive, low sample volume assays. The utilization of dried blood spot sampling for biomarkers can certainly facilitate the collection and storage of test samples [41]. Multiple mechanistic biomarkers of interest, or biomarker and drug measurements, may need to be multiplexed into a single assay method to adequately cover peak/trough analyte levels. In addition, in order to account for intra- and intersubject variability in drug effect at each time point, it is important to characterize the entire biomarker sampling interval with and without drug on board.

Variation in biomarker levels from time to time, be it from reproducible rhythms (circadian/diurnal), food effects, disease state, and so on, make it critical that the placebo biomarker response profile is understood so that changes in the biomarker during drug intervention are not attributed to drug activity when they are really just biological variability. It may be necessary to draw predose samples from each subject if significant intersubject biomarker variability exists at the beginning of a study. Finally, proper sample collection and handling strategies, beyond those assessed as part of an analytical method validation, must be evaluated to determine their effect on biomarker variability. Matrix (e.g., plasma or serum), type of blood anticoagulant, immediate sample freezing or processing, platelet inactivation, and tissue or cell lysis protocol are some of the types of variables that need to be considered for biomarkers and implemented in a PK/PD study design.

20.6 ASSAY AND DATA QUALITY CONSIDERATIONS FOR PK/PD BIOMARKERS

As previously described, a biomarker can be a biochemical, physiological, imaging, or behavioral characteristic that is objectively measured as an indicator of a normal or pathologic process or a response to therapeutic intervention. In the context of this chapter, the primary focus is on biochemical markers. Biochemical markers can be characterized by a known chemical formula and structure and can belong to various compound classes, such as steroids, lipids, phospholipids, carbohydrates, nucleotides, peptides, proteins, and so on. The two primary methodologies for the identification of biochemical biomarkers are metabolomics and proteomics. Each of these comprises a comprehensive set of study designs and analytical technologies often in comparative studies between normal versus diseased and treated versus untreated study groups to identify differentially expressed biomarkers. Metabolomics primarily focuses on nonprotein, nonpeptide biomarkers and utilizes various sample extraction protocols followed by liquid chromatography (LC) and/or gas chromatography (GC) separation technologies combined with MS and/or nuclear magnetic resonance (NMR) detection. Proteomics, on the other hand, focuses on the identification of peptide and protein biomarkers using separation methods amenable to resolving larger molecular entities, including 2D gel and capillary electrophoresis, reverse phase high performance liquid chromatography (RP-HPLC), most often followed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or electrospray ionization (ESI) LC-MS/MS detection. Once the biochemical biomarkers are identified, assays can be developed for their quantification. The two most often used technology platforms for this in body fluids and tissue extracts are immunoanalytical, or ligand-binding, assays and more recently, LC-MS/MS with the combination of immunocapture sample enrichment, or IMM-LC-MS/MS. This section provides information on the factors that determine which platform to utilize for biomarker quantification and discusses the key challenges of biomarker assay validation using these technologies.

20.6.1 Technologies for Biomarker Measurements

A variety of factors influence which technology platform should be used to develop a biomarker assay. These factors include technical feasibility, fitness for

purpose/validatability, translatability between nonclinical and clinical settings, throughput, availability at contract research organizations (CROs) to support larger scale clinical studies, and time required versus time available for method development.

Technical feasibility considerations should access the selectivity and sensitivity of the method to accurately and precisely quantify the biomarker in the biological matrix relative to the biological variability and expected modulation of the biomarker. Biomarker concentrations with a significant modulation window between normal and diseased states and low intra- and intersubject variability can be differentiated using a method with greater variability, while highly variable biomarkers with a narrow difference between normal versus disease states have limited chance to be differentiated unless the method variability is minimized. Figure 20.5 illustrates this point for results obtained from a nonclinical study where animals were administered a statin drug and plasma mevalonate levels were monitored by LC-MS/MS. The modulation window between the low and high dose groups is a combination of the difference between the individual subject variability and the established assay accuracy.

Enzyme-linked immunosorbent assay (ELISA) and LC-MS/MS assay have inherently different selectivity and specificity based on the analytical principles they employ. The selectivity of a method is the measure of its ability to determine a particular analyte in the presence of other interfering components in the matrix. A method that is perfectly selective for an analyte is specific [42,43]. The inherent selectivity of immunoanalytical or ligand-binding assays, such as ELISA, is based on the selectivity of the antibody–antigen interaction. Antibodies are heterogeneous in their antigen specificity, which is attributed to the polyclonal B cell response that leads to their formation. This known heterogeneity, combined with variable affinity to the antigens, can cause considerable variability in the specificity and sensitivity of immunoassays.

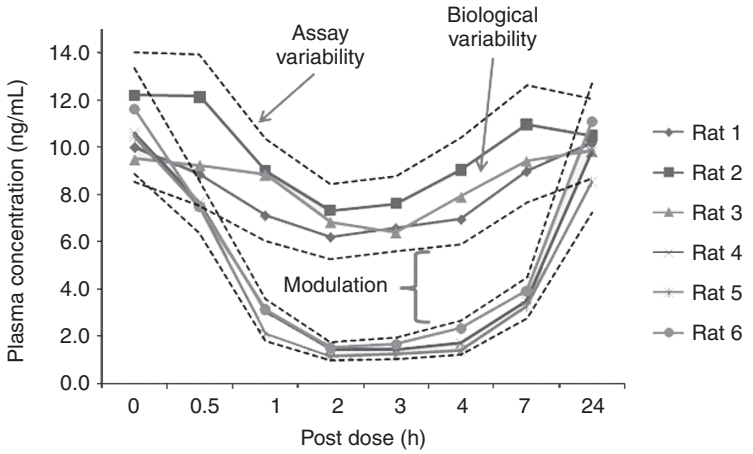

Figure 20.5 Rat plasma mevalonic acid concentrations measured by LC-MS/MS following oral administration of a statin (rats 1–3: 10 mg/kg; rats 4–6: 100 mg/kg). Variability in a biomarker measurement comes from both technical and biological sources. Assay variability must be minimized when developing a method in order to accurately determine the modulation window of a biomarker in different states (i.e., healthy vs disease, treated vs untreated). The established overall accuracy error of the assay was 15% (represented by the dotted lines).

The three main contributors to the selectivity of an LC-MS/MS method are (i) sample preparation, including directed extraction of the analyte from interfering components; (ii) LC separation; and (iii) selected reaction monitoring (SRM) detection based on the precursor and product ion mass-to-charge (m/z) values. Thus, LC-MS/MS assays tend to be more specific than immunoassays. On the other hand, LC-MS/MS assays are limited to analytes with molecular weights below ~10 kDa, the m/z range detected by contemporary instrumentation. Most commonly used platforms are triple quadrupole mass analyzers with m/z ranges <4000. Using an ESI source that is capable of producing multiply charged ions can potentially bring small proteins within the useful m/z range of a triple quadrupole mass spectrometer. Other mass analyzers, such as time-of-flight (TOF) instruments, have a higher m/z range. However, they are often combined with ion sources, such as matrix-assisted laser desorption/ionization (MALDI), that are not amenable for quantitative measurements. Gas chromatography-mass spectrometry (GC/MS) can also be used for quantification of low molecular weight, highly volatile compounds.

A fundamental challenge in developing and validating robust quantitative biomarker assays, in contrast with assays for drug measurements, is the lack of relevant biological matrix that is devoid of the analyte(s) of interest. This not only requires an assessment of assay selectivity but also necessitates a strategy for preparing assay calibrators. Often a substitute calibrator matrix cannot be identified because of interfering components or because of inadequate analyte recovery compared to the biological matrix of interest. An alternative approach to remedy this issue that has been successfully applied to MS-based methods is the use of a stable-isotope-labeled isoform of the biomarker compound as the calibrator [44]. The incorporation of C13, N15, 2H, or other heavy elements into the synthetic biomarker allows it to maintain similar recovery, chromatographic, and ionization properties as the endogenous form but provides the shift in mass to be resolved on the mass spectrometer.

In terms of sensitivity, ligand-binding ELISAs routinely reach 1–0 pg/mL sensitivity, especially using electrochemiluminescence (ECL) or fluorometric detection. LC-MS/MS assays have similar sensitivity in the 1–10 pg/mL and low nanogram per milliliter range depending on analyte responses and matrix effects. More recently, immunoaffinity enrichment using analyte-specific antibodies has been coupled with LC-MS/MS analysis to increase the sensitivity [45]. There are also significant differences in the precision and accuracy of the various platforms. Immunoassays usually have greater variability because of their simple sample preparation procedures that often use straight, or minimally, diluted plasma or serum, and have inherent specificity and potential cross-reactivity issues. Commonly, for validated ELISA assays, ±20% accuracy and precision with <30% total error is acceptable. LC-MS/MS assays, on the other hand, use more elaborate sample preparation procedures and utilize an internal standard to correct for process and analysis variability and, therefore, are usually validated within ±15% accuracy and precision criteria. The main characteristics of various assay technologies that can be used for biomarker quantification are shown in Table 20.2. Besides the technical considerations listed above, some logistical consideration such as typical development time lines are also included.

20.6.2 Fit-for-Purpose Assay Validation Considerations for PK/PD Studies

While biomarkers are increasingly used in internal decision making within pharmaceutical companies, there is a focused interest in their utilization for regulatory evaluation

TABLE 20.2 Comparison of Assay Characteristics for Commonly Used Quantitative Biomarker Assay Technologies

Assay Characteristic	Ligand binding/Immunoassay	LC-MS/MS
Selectivity	Wide range of analytes Favored method for large MW proteins On the basis of antibody–antigen interaction Can measure multiple analytes nonselectively that cross-react with capture/detection antibody Can be multiplexed	Applied for <10 kDa MW polar/ionizable analytes On the basis of HPLC separation, molecular weight and fragment ion m/z values in selected reaction monitoring (SRM) detection Typically measures one specific analyte/SRM channel at a given retention time, can be multiplexed
Sensitivity	in picogram per milliliter	in picogram per milliliter to nanogram per milliliter
Precision (%CV)	<20	<15
Accuracy (%RE)	<20	<15
Throughput	Sample preparation in automated parallel processing, incubations: few hours to overnight/96-well plate Reading time: <5 min/plate	Sample preparation in automated parallel processing in <30–120 min/96-well plate LC-MS/MS analysis time: 2–15 min/sample
Sample preparation	Usually none, direct analysis of serum and plasma	Extraction, addition of stable-isotope-labeled internal standard
Reagent needs	Antibody reagents are needed; Labeling of reagents are required (source of variability)	Usually no specific reagents are needed; Stable-isotope-labeled internal standards may need to be synthesized
Equipment and sample analysis cost	Equipment cost is <$100, 000(~$60/sample)	Equipment cost is <$500, 000(~$100/sample)

Abbreviations: MW, molecular weight; CV, Coefficient of Variation; RE, Relative Error.

of new drug candidates. The FDA Critical Path Initiative outlines the framework and evidence needed to qualify biomarkers for regulatory drug evaluation purposes and defines some of the critical biomarker needs in various disease areas [19]. This need for high confidence in biomarker data used in scientific or regulatory decision making emphasizes the need for high quality, reproducible, and reliable assay measurements that are translatable between nonclinical and clinical applications and validated for their intended purpose. In a recent conference, the American Association of Pharmaceutical Sciences (AAPS) Clinical Ligand Assay Society Biomarkers Workshop addressed the key challenges in biomarker research and summarized validation recommendations for immunoanalytical ligand-binding biomarker assays in a summary report [46]. Follow-up publications continued to discuss the need for iterative, fit-for-purpose approaches to biomarker method development and validation keeping in mind the intended use of the data, as well as the regulatory requirements associated with that use [43,47].

The fit-for-purpose concept advocates the progressive rigor employed for biomarker assays from initial characterization and qualification of assays to fully validated methods. Some of the key concepts related to this are described in the sections below and summarized in Table 20.3.

20.6.3 Assay Validation Versus Biomarker Validation

Regardless of which purpose the biomarker is used for, its successful utilization in scientific decision making requires its validation for the intended purpose. This fit-for-purpose validation consists of two critical steps: (i) the technical validation of the analytical method used to quantitatively measure the biomarker and (ii) the biological validation of the biomarker, confirming its linkage to the relevant biological and pharmacological hypothesis that is being tested. These two validation steps often occur concurrently, since a reliable analytical assay needs to be developed first to be able to test the biomarker's linkage to pharmacology in an animal model or human. Once the biomarker linkage to biology is tested and modulation of the biomarker between normal versus disease state or response to a therapeutic intervention has been shown, the analytical method requirements can be finalized and the method validated. Depending on the drug discovery program, multiple potential biomarkers in multiple animal models and species may be under investigation until a decision-making biomarker is selected. The development of multiple biomarker assays in multiple species and matrices and their testing in relevant disease models and clinical populations is a resource-intensive process with respect to both cost and time. Therefore, carefully designed and robust strategies for the development and fit-for-purpose validation of translatable biomarkers between nonclinical and clinical applications need to be formulated in the form of a biomarker research and operating plan [17,18]. The existence of this research plan containing input from nonclinical and clinical biology, pharmacology, PK, analytical, and PK/PD scientists can assure timely availability of validated biomarkers and assays to support nonclinical and clinical decision making.

20.6.4 Technology/Methodology Translation Considerations for PD Markers

The translatability of methods from the nonclinical to clinical application, including sample collection and preparation, is an important consideration for any biomarker—including PD markers. While it is not uncommon and often helpful to determine biomarker levels in the target tissues at the site of action in nonclinical models, the clinical applicability of these is limited because of the invasiveness of sample collection. In these cases, it is helpful to characterize the correlation between the tissue concentrations and circulating biomarker concentration in plasma, serum, or secreted concentrations in urine to determine if sampling from a less invasively obtained biological matrix can replace the tissue sample in the clinical setting. Other aspects for clinical translation are availability of the technology at CROs and throughput and validation requirements.

20.7 CONCLUSIONS/FUTURE CHALLENGES

The identification, analysis, and application of biomarkers in the development of therapeutics have come a long way over the past decades. The information provided by

TABLE 20.3 Characteristics of Fit-for-Purpose Ligand-Binding and LC-MS/MS Biomarker assays for Applications in Drug Development

	Drug Development Stage and Purpose of Biomarker Assay		
	Discovery	Nonclinical Development	Clinical Development
Assay Characteristic	• Identification of biomarker candidates • Use biomarkers for target validation • Evaluate biomarker response in animal model	• Validation of the biomarker's linkage to the disease state and/or mechanism of action • Characterization of the efficacy and safety in animal model • Drug candidate selection	Validation of the biomarker in human, for purposes of • Clinical trial go/no go decisions (mechanism, compound efficacy) • Clinical trial dose range determination (PK/PD) • Clinical trial design (length, size of population, powering of studies) • Compound differentiation • Disease diagnosis and prediction • Surrogate end points • Treatment decisions
Reference standard	Consistent—source determined	Stability as reference material and in biological matrix	Well-characterized (identity, purity) stability established, inventory/supply established
Assay dynamic range, LLOQ and ULOQ	Estimation based on endogenous levels and expected modulation	Verification based on incurred samples in animal studies, and in human, if feasible, if human assay is needed Establishment of LLOQ and ULOQ in multiple validation runs	Verification based on incurred samples Establishment of LLOQ and ULOQ in multiple validation runs
Selectivity and specificity	Determination based on reagent supplier or literature data, assess matrix effects, and potential interferences Determination of standard curve matrix (or surrogate matrix)	Verification of selectivity, specificity, and matrix effect in method validation runs	Verification of selectivity, specificity, and matrix effect in method validation runs

Precision and accuracy	Establishment of expectations for required accuracy and precision based on biomarker variability and expected modulation window	Determination of accuracy and precision in method validation runs (multiple runs for interassay values)	Determination of accuracy and precision in method validation runs (multiple runs for interassay values)
Recovery	Determination of recovery from biological matrix, establishment of expectations for desired sensitivity and robustness	Validation of recovery in spiked quality control samples and incurred samples	Validation of recovery in spiked quality control samples and incurred samples
Dilution linearity/ parallelism	Verification if exist in incurred and spiked samples	Validation in spiked and incurred samples	Validation in spiked and incurred samples
Sample collection, processing, and storage stability	Establishment of feasibility, evaluation of stability at bench-top and in process	Establishment of bench-top and short-term stability, freeze/thaw, if used	Establishment of freeze–thaw and long-term stability

biomarkers to elucidate the drug target, biochemical mechanism, or disease outcome can be used nonclinically to predict efficacy and safety in humans. Therefore, biomarkers provide the cornerstone for PK/PD modeling and translational research. For proper design of PK/PD studies, biomarkers and the analytical methods used to measure them, require the degree of validation necessary to confidently answer the question the experiment was designed for. Technology also is advancing to the point where not only robust analytical methods for a PD biomarker can be established but mutianalyte and multiparameter assays ("-omics," arrays, etc.) can also be developed to understand the PD system as a whole. Similarly, while some pharmaceuticals modulate well-established pathways and biomarkers, and can be applied to a population generally, such as statins, others require biomarkers to enable the selection, or stratification, of certain patient populations to demonstrate efficacy. While this systems pharmacology approach promises to lead to the design of better drugs with the right dose in the right patient, the amount of data generated and the associations that can be revealed are daunting. Therefore, bioinformatics will be an essential and integral part of future drug development to process and interpret large amounts of data. As biomarkers at the metabolome, genome, and proteome levels continue to be discovered, validated, and employed both for new drug development and in improving the utility of existing therapeutics, the next decades will no doubt bring more effective and safer therapies to patients.

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