

From Traditional Biomarkers to Transcriptome Analysis in Drug Development

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Abstract: Traditional biomarkers have played an important role in drug development as well as patient care. A single traditional biomarker or surrogate endpoint is unlikely to either characterize the complete pathophysiology of a complex disease or capture all the therapeutic benefits or potential adverse effects that a drug will have in a diverse patient population. Transcriptome analysis, on the other hand, can provide a large-scale survey of gene expression associated with the etiology of a human disease or pharmacological responses to a therapeutic intervention. The quantitative and qualitative readouts can provide increased power to identify novel drug targets or biomarkers indicative of drug safety or efficacy. Transcriptomics has positively impacted drug development and will continue to improve the medicines of the future. Here, we describe the increasingly important roles that traditional biomarkers and transcriptome analysis have played in various phases of drug discovery and development as well as the opportunities and challenges that they present to the pharmaceutical industry.

INTRODUCTION

Today, it is widely recognized that most drugs are only effective in 40 to 60% of the patients for whom they are prescribed, and that the potential for adverse effects is a serious concern in drug development. As drug development moves into the next decade, there are increasing expectations that medicines will be personalized with increased efficacy and reduced risks of adverse events. As a result, there will be pressure to intensify the search for novel targets for drug development as well as new biomarkers that can predict the likely response (i.e. benefit or harm or the lack thereof) to the drug being developed. Well suited to face these challenges are genome-wide scanning technologies that can be used to associate genes with a disease or drug intervention. One such technology is transcriptome analysis, which allows profiling of the subset of genes transcribed in a given organism, or the transcriptome, that provides a dynamic link between the genome, the proteome and the cellular phenotype. Among the extremely powerful techniques used in transcriptomics are DNA-microarrays, which allow determination of the mRNA expression level of practically every gene of an organism.

Transcriptomics was formally introduced in the early 1990's [1-4], and now is just one of the many "omic" technologies introduced that allow mass arrayed probing of multiple targets. In its infancy there were many different materials used to "array" and probe the transcriptome which did not provide equivalent readouts. Present day transcriptomics has incorporated novel advances in chemistry for both

solid surface arrays and probes, as well as developing a formal set of guidelines for annotation and reporting through minimum information about a microarray experiment or MIAME [5] thus bringing more homogeneity to its use in clinical applications and drug development.

In recent years, the high rate of drug attrition is pushing up the cost of drug development, estimated to have increased at an annual rate of 7.4% above inflation [6]. The most costly process within drug development and the most to suffer from attrition is the Phase III "outcome" trials that evaluate clinical benefit and safety in a disease population. Not surprisingly, more and more pharmaceutical companies are taking the first steps to develop complex drug registration packages that include epidemiologic, therapeutic and/or pathophysiologic data which include biomarkers and transcriptome analyses.

A biological marker or biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Table 1) [7, 8]. Zolg and Langen [9] recently characterized a biomarker as a molecule that indicates an alteration of the physiological state of an individual in relation to health or a disease state, drug treatment, toxin, or other environmental challenges. A biomarker can be a physical or imaging measurement (e.g. weight, color, pressure, intimal medial thickness) or a chemical (e.g. metal) or biological (e.g. glucose, proteins) entity.

Biomarkers that have been used extensively over the past decades to describe both normal and pathological conditions include: an elevated level of urine human chorionic gonadotropin (hCG) as a measure of normal pregnancy, a disproportional

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Table 1. Definitions of biomarkers.

Term	Definition
Biomarker	<ul style="list-style-type: none"> A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [7]
Clinical end point	<ul style="list-style-type: none"> A characteristic or variable that reflects how a patient feels or functions, or how long a patient survives [7]
Surrogate end point	<ul style="list-style-type: none"> A biomarker that is used in therapeutic trials as a substitute for a clinical endpoint and is expected to predict the effect (i.e. benefit or harm, or its lack thereof) of the therapy [7, 8]

body weight as a primary determinant of obesity, and increased blood pressure and cholesterol levels as hallmarks of cardiovascular risks. Other examples of more esoteric biomarkers include tumor size in cancer survival, bone density in osteoporosis and viral load in infectious diseases. Advances in technology have allowed the incorporation of measurements of many biomarkers in the regimen of patient care without increased risk to patients. In fact, there is hardly any disease from infection to oncology that is treated nowadays without testing "biomarkers". Some biomarkers can discriminate normal from diseased states (i.e. diagnostic biomarkers), while others predict the likely course of disease progression (i.e. prognostic biomarkers) or response to therapy (i.e. stratification biomarkers) or drug effect (pharmacodynamic or PD biomarkers) (Table 2). However, the most utilized application of biomarkers is for internal decision making along the drug development pipeline. These measurements can provide information on optimizing dose, drug efficacy, and helping define the patient profiles that will benefit the most from the specific therapeutic intervention.

Significant correlations between biomarkers and benefits in clinical outcomes trials that are identified (and included in the regulatory dossiers) will be used to build the infrastructure to enable more biomarkers to be accepted as surrogate endpoints of disease/benefit by the FDA and other regulatory agencies. One such biomarker that has attained the status of a surrogate endpoint is plasma cholesterol. Quantitative changes in cholesterol have been used as the benchmark for measuring increased clinical benefit of newly introduced members of the statin drug class (e.g. Lipitor™). AstraZeneca, Merck, Pfizer, Bristol-Myers-Squibb, the manufactures of various statin drugs, all conducted studies associating the reduction of cholesterol level to the efficacy of their drugs [10].

Transcriptomics has been widely used in drug discovery and development to discover genes that are associated with specific diseases, to classify diseases, to identify drug targets and annotate gene functions. The increased efforts by both pharmaceutical and biotechnology industries in this area have resulted in significant progress in the technology developed and utilized for biomarker

Table 2. Characterization of biomarkers.

Type	Characteristics	Sources of Biomarkers	Key Validation Requirements
Diagnostic biomarkers	<ul style="list-style-type: none"> Discriminate normal from a specific disease or disease state independent of a specific therapy 	<ul style="list-style-type: none"> Expression analysis Disease mechanism studies Epidemiology studies 	<ul style="list-style-type: none"> Correlation with a specific disease or disease state
Prognostic biomarkers	<ul style="list-style-type: none"> Predict the likely course of disease progression independent of a specific therapy 	<ul style="list-style-type: none"> Expression analysis Animal models Epidemiology studies 	<ul style="list-style-type: none"> Correlation with a clinical outcome independent of a specific therapy
Stratification biomarkers	<ul style="list-style-type: none"> Identify patients likely to respond to a specific drug or suffer from its side-effects prior to administration of the drug 	<ul style="list-style-type: none"> Pre-clinical studies Clinical trials Epidemiology studies 	<ul style="list-style-type: none"> Correlation with a clinical response (benefit/harm) upon administration of the drug in controlled clinical trials
PD/PK Biomarkers	<ul style="list-style-type: none"> Correlate response to a specific drug with concentrations of the drug or its metabolites 	<ul style="list-style-type: none"> Drugs or metabolites Animal models 	<ul style="list-style-type: none"> Correlation with the concentration or activity of a specific drug in animal and human studies
Efficacy Biomarkers	<ul style="list-style-type: none"> Monitor the beneficial effects of a specific drug on the intended drug target or medical condition 	<ul style="list-style-type: none"> Molecular targets or downstream molecules Clinical trials 	<ul style="list-style-type: none"> Correlation with the concentration or activity of a specific drug in clinical trials with placebo controls
Toxicity Biomarkers	<ul style="list-style-type: none"> Monitor the adverse effects of a specific drug on any unintended cellular processes, cells, tissues or organs 	<ul style="list-style-type: none"> Histopathology Clinical chemistry Toxicology studies Clinical trials 	<ul style="list-style-type: none"> Correlation with the concentration or activity of a specific drug in clinical trials

discovery. Following is an integrative description of biomarker use in the various phases of drug development and considerations for incorporation of this new tool in the multiple phases of drug development.

USE OF BIOMARKERS IN DRUG DEVELOPMENT

Biomarkers predictive of drug toxicity or efficacy have conceivably the greatest impact on successful drug development. However, only a small number of biomarkers can accurately predict clinical safety and efficacy endpoints so as to be considered as surrogate endpoints as defined (Table 1) [7, 8]. On the other hand, biomarkers indicative of the mechanism of action of drug intervention can provide great predictive value in early drug development even if they do not become surrogate endpoints. The major challenge for drug development is how to select candidate biomarkers for a compound and differentiate surrogates from other biomarkers. Clearly, the choice is largely dependent upon the stage of drug development as well as the availability of resources. In addition, the biological materials that can be easily obtained from human subjects consenting to first-time-in-human trials are often limited. Therefore, the emphasis on selecting appropriate candidates should be heavily placed in the early stages of the drug discovery pipeline where both the costs of evaluating many candidate biomarkers and the risk of moving compounds forward that have adverse or toxic effects are reduced. The functions of biomarkers that can be used for pharmacodynamic evaluation, toxicology determination, candidate selection, patient stratification, identification of new indications for existing drugs and even as early diagnostics or predisposition tests to expand the drug market to pre-symptomatic individuals can help bring clarity to understanding the compounds being developed and therefore significantly enhance researchers' capability of decision making, including attrition of drug candidates. The use of biomarkers during the various stages of drug discovery and development are summarized in Table 3 and described here as examples of how transcriptome analysis may be applied in the future to enhance the discovery and development of novel pharmacophores.

Pre-Development Stage

Identification and validation of a target or targets for therapeutic intervention constitutes the initial step in drug discovery. At this pre-development stage, normal and pathological processes are studied at the cellular and molecular levels, which provide a list of potential targets. Their clinical relevance is then established in the ensuing correlative studies, in which the potential targets for intervention or the molecules directly controlled by the potential targets are evaluated along with traditional biomarkers of diagnostic and prognostic significance. For example,

the HER2/neu protein was initially identified as an oncogene based on molecular studies *in vitro* and in animal systems [11, 12]. Its clinical significance for cancer was subsequently established with a correlative clinical study, in which its overexpression or gene amplification was linked to lymph node involvement [13]. A cancerous lymph node is considered a traditional surrogate biomarker indicative of poor patient prognosis. HER2/neu has now become one of the most important targets for the treatment of human breast cancer [14-16]. In addition, it has been frequently targeted for drug intervention along with other signaling molecules that play important roles in the pathogenesis of human breast cancer [17, 18].

Table 3. Utilities of biomarkers in drug development.

Stage of Drug Development	Potential Uses of Biomarkers
Pre-Discovery	<ul style="list-style-type: none"> • Study disease mechanisms
Discovery	<ul style="list-style-type: none"> • Define drug targets • Explore mechanisms of action for the compound class • Establish structure-activity relationship
Pre-Clinical	<ul style="list-style-type: none"> • Build pharmacokinetic-pharmacodynamic models • Highlight mechanisms of drug action • Establish safety and efficacy end points • Guide compound selection and retention
Early Clinical	<ul style="list-style-type: none"> • Demonstrate bioavailability and bioequivalence • Determine dose response • Confirm mechanisms of drug action in humans
Late Clinical	<ul style="list-style-type: none"> • Define targeted population • Allow dose selection and optimization • Use for registration
Post-Marketing	<ul style="list-style-type: none"> • Allow product differentiation • Stratify patients • Monitor therapeutic response • Monitor side-effects

It has been estimated that the whole pharmaceutical industry has so far identified only 10-14% of the roughly 3,000 "druggable" targets present in human genome [19, 20]. Transcriptomics offers the greatest impact in target discovery by profiling the expression patterns of thousands of genes in complex biological mixtures. The clustering profiles provide a signature that can be compared with future transcriptome assessments establishing association of the novel genes with the disease. In fact, new approaches for target discovery for oncology now are characterized by profiles using panels of biomarkers that evolve from mainly transcriptomic arrays [21-31 and see the papers by Burczynski *et al.* Dracopoli, and Wadlow & Ramaswamy in this issue]. For example, gene expression profiles can not only stage and classify breast cancers more accurately than traditional histopathological evaluation, but can also predict their clinical outcomes [21, 28-30], implicating the

involvement of this collection of genes in the etiology of the malignancy that could potentially be targeted for therapeutic intervention. A recent large-scale meta-analysis of cancer microarray data identified 2 common transcriptional profiles, one that is a characteristic of neoplasms as compared to their normal counterparts and the other that is a common feature of various types of undifferentiated cancers [31]. The fact that expression patterns rather than individual genes characterize cancers attests to the critical role that transcriptomics can play in target identification and biomarker discovery at the preclinical stage. With the advancement in technologies such as proteomics and transcriptomics, a wave of novel drug targets and biomarkers have emerged, and their acceptance as viable drug targets or surrogates will undoubtedly require extensive correlation with clinically validated and well established markers of disease. Clearly, the validity of the target(s) and the success of the drug development programs depend upon successful translation of basic research findings from the new disease profiling technologies, including transcriptomic analysis, into discovery of targets that can then be applied to tumor typing and eventual stratification of patient to selected therapies.

Drug Discovery Stage

Once a drug target is associated with a specific disease and intervention of the target is proposed to lead to a desired change in the targeted tissues and cells, thousands of compounds must be synthesized and screened against the target in order to find compounds with the desired biological and pharmacological properties. Targets associated with mechanisms of drug action are often used in these initial high-throughput biological assays. For instance, inhibition of kinase activity is measured during screening for potential kinase inhibitors instead of cell growth or proliferation rates, which may be used later to further characterize the activity of the selected lead compounds. Measurements of clinical endpoints or surrogate endpoints are invariably impractical and unnecessary at this point in the drug progression pipeline. However, understanding of the role of targets in disease pathology and their interactions with other biomolecules, including nucleotides, proteins and carbohydrates, is critical in the discovery of the lead compounds for further development based on the chemical structures and their biological activity relationships.

Pre-Clinical Stage

The development of new drugs requires a full understanding of the potential toxicity, safety, and pharmacokinetics of these new agents in animal models prior to entry into man. Pharmacologic and toxicological effects of the new drugs on virtually all body systems must be documented. In addition, the carcinogenic potential of the new drug and its

potential hazard to reproductive function and fetal development need to be determined in long-term studies. Initial toxicity evaluations follow administration of a single dose of the new drug, while subsequent tests involve multiple doses over longer periods of time. In general, tissues, organs and blood components from 2 or more animal species, including one rodent and one non-rodent species, are evaluated to establish the profiles of general and end organ toxicity in relationship to dose. In such typical preclinical animal studies, both the compounds under development and their metabolites are also measured to establish when and how the drug may be changed or eliminated in the body. In addition to these traditional assessments, novel cellular transcriptome-based methods are also being employed to evaluate downstream toxicity and pathophysiological changes induced by in-life-testing phases of the candidate drugs. Laser capture microdissection (LCM) followed by cell specific transcriptome analysis of selected organs/cells will expand the survey of efficacy, drug toxicity and increase surveillance of organ damage at an earlier stage of the pipeline than allowed by traditional biomarker readouts based on standard pharmacokinetic/pharmacodynamic (PK/PD) relationships. LCM and similar tools will help to pick the winners as we move from preclinical arena into the clinic.

Drug side-effects are common problems usually identified in longer term/repeat dose studies, but the mechanisms responsible for diversity of drug toxicities are largely unknown. The use of novel genomic approaches (e.g. transcriptomics, proteomics) to define the molecular profiles associated with adverse effects of a compound has created a new multidisciplinary science in toxicogenomics [32-36]. It greatly facilitates the identification of novel biomarkers for predictive toxicology and mechanism-based risk assessment. New biomarkers of drug toxicity and their underlying mechanisms can be uncovered by examining changes in gene expression profiles in cells or tissues in response to the drug. The potential for a new drug to be toxic may be predicted based on the similarity of the pattern of gene expression changes that it elicits in *in vitro* or *in vivo* systems to those identified for a wide variety of toxic compounds.

Toxicogenomics is rapidly becoming a standard analysis in toxicology studies, and could impact all stages of drug safety evaluation. It also has the potential to identify and characterize the molecular mechanisms that lead to drug-specific toxicity in animals and humans so one can extrapolate the toxicity results from animals to humans and reduce numbers of adverse drug effects [37]. It is conceivable that molecular biomarker profiles or signatures could be increasingly used to support and eventually to supplant the laborious and subjective histopathology evaluation of the safety of a developing compound. Undoubtedly, continuing progress in analysis and validation of gene profiles in

response to various drugs or toxins will help to realize the full potential of toxicogenomics in drug safety assessment.

It should be pointed out that while approximately 16% of drugs under development fail due to adverse effects, many more (46%) fail in clinical development due to lack of efficacy [38]. The fundamental question for the development of any drugs is whether the drug is efficacious for the intended indications at tolerable doses. An earlier and clearer answer to this question poses the most serious challenge to the pharmaceutical industry, who can neither afford to further develop a drug with little efficacy, nor justify premature termination of a potentially effective drug. Therefore, incorporation of mechanism-based PD biomarkers indicative of the impact of new drugs on the molecular target for intervention would provide early evidence of purported efficacy in relation to the kinetics, safety and toxicity of the drugs. Tomaszewski [39] recently provided such an example in which the development of a proteasome inhibitor PS-341 (Velcade™) benefited greatly from the use of 20S inhibition assay in white blood cells as a mechanism-based efficacy biomarker.

Transcriptomics may also play a small predictive role in identifying the most efficacious drug candidates and teasing out appropriate drug combinations based on their gene expression profile. For instance, many pharmaceutical companies including Pfizer, GlaxoSmithKline, Merck and others have ongoing transcriptomic studies to correlate gene expression with drug activity of a broad range of compounds. Examples include Microcystin-LR, Carbon tetrachloride, and Etoposide at Pfizer [40], clofibrate and 3-Methylcholanthrene at Merck [41] and Interferon- α at Roche [42]. Similarly, the National Cancer Institute of the National Institutes of Health has profiled the gene expression patterns of 60 human cancer cell lines in response to 1400 compounds in an effort to establish the molecular mechanisms of sensitivity or resistance to the clinical agents such as 5-fluorouracil and L-asparaginase [43]. In addition, drugs, which act cooperatively, synergistically or antagonistically on certain critical targets that will effect patient treatment and welfare (i.e. liver function tests), can be probed with transcriptomics analysis technologies in preclinical model systems. Undoubtedly, predictive knowledge before launching large phase clinical trials would enable the best compound in a series to be identified earlier in the clinical development plan, thus culling out those with potential negative effects when administered with other concomitant medications.

Early Clinical Stages

Initial introduction of an investigational new drug into a small number of patients or healthy volunteers in Phase 1 clinical trials (i.e. first-time-in-human or FTIH and subsequent clinical pharmacology studies)

allows evaluation of its tolerance at different doses, definition of its pharmacologic effects at anticipated therapeutic levels, and establishment of its absorption, distribution, metabolism, and excretion patterns in humans. Along with the determination of the pharmacokinetic and pharmacological effects of the drug, the mechanism of action of the drug in humans may also be evaluated to gain early evidence on its effectiveness. The ensuing early controlled Phase 2 clinical studies (i.e. IIa, IIb), with typically no more than several hundred subjects, will determine the efficacy of the drug for a particular indication or indications in patients with the disease or condition as well as the common short-term side effects and risks associated with the drug.

The most widely used biomarkers in these first set of assessments in patients include safety parameters such as heart rate, blood pressure, electrocardiogram parameters, clinical chemistry, hematology, coagulation markers, and the plasma concentrations of drugs and their metabolites to document the rate of absorption and excretion from the body. These safety and PK parameters establish the duration of exposure to the drug and serve as a guide for safe dose selection and escalation. These assessments also help to establish bioavailability and bioequivalence profiles of the drug in any new form, such as in a new formulation (e.g. composition of inactive excipients, flavoring or coloring), as well as evaluation of new doses or new routes of administration (e.g. from injection to oral dose form).

The more recent trend in the pharmaceutical industry to develop "targeted therapies" significantly increases the needs to acquire such understandings of biomarkers and their therapeutic relevance. The discovery of the important roles of members of the human epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinases in a number of solid tumors results in a series of new targets. Many pharmaceutical companies are targeting EGFR kinases to develop treatments for such tumors including breast cancer and non-small cell lung cancer [14-16,44-47]. In addition, biomarkers that identify the patient population that will benefit from the targeted therapies (targeted population) are also essential to assure the success of such therapies.

Biomarkers of potential use for stratification of patients include the target receptor or enzyme for drug intervention and modalities that can confer resistance to the drug. For example, HER2/neu overexpression or gene amplification was a part of the eligibility criteria for clinical trials on trastuzumab, a humanized monoclonal antibody against HER2/neu that has been subsequently approved for the treatment of metastatic breast cancer [14-16]. However, one should be mindful of the risks associated with the use of the targets as stratification biomarkers. For instance, Iressa, an inhibitor of the EGFR, was developed for the treatment of human non-small cell lung cancers that

frequently overexpress the receptor tyrosine kinase [44-47], but tumor response to Iressa is not predictable based on tumor EGFR membrane staining alone [48]. Use of the target for patient stratification would have never allowed the development of estramustine, one of the most effective prostate cancer therapies that was designed as an alkylating agent targeted at estrogen receptors (ER), but actually exerts its antitumor effects *via* ER-independent antimetabolic activity [49]. Similarly, BAY 43-9006, a new drug candidate designed as a *Raf* inhibitor, has been shown to inhibit not only cell proliferation mediated by the RAF/MEK/ERK pathway but also tumor angiogenesis mediated by VEGFR-2 and the PDGFR tyrosine kinases [50]. Thus, analysis of *Raf* inhibition may not truly reflect the antitumor potential of the drug candidate.

Traditional biochemical biomarkers of potential use for monitoring patients on therapy are generally molecules downstream from the target for drug intervention (e.g. cholesterol for statins [51-53]) or surrogate endpoints of a clinical outcome (e.g. CD4 counts in HIV infection [54, 55]). New biomarkers may also come from enhanced use of both invasive and non-invasive imaging tools in clinical patient assessments. Multiple imaging modalities have been implemented to monitor the responses to therapeutic interventions in patients with coronary atherosclerotic disease [56-58], multiple sclerosis [59], solid tumors [60] and pain [61, 62], among others [63]. Thus, both biochemical and imaging biomarkers may help to expand the list of surrogate biomarkers. Appropriate use of validated surrogate endpoints that can predict clinical benefit could expedite the clinical trials by shortening the duration that patients have to be monitored.

All drug companies face dilemmas concerning the utility of biomarkers in early clinical studies. On one hand, research at the pre-development and pre-clinical stages could provide a list of candidate biomarkers for stratification of patients based on efficacy or safety concerns or for monitoring of patient response based on the mechanisms of action. On the other hand, few, if any, of these biomarkers may have been causally associated with drug efficacy or safety in humans. Incorporation of a large number of poorly validated putative biomarkers in early clinical trials adds to the cost of the trials and could be futile if the drug fails to demonstrate adequate activity. Inclusion of large survey tools like microarrays may provide a better means of exploration of putative biomarkers for downstream success but should be limited to developmental, preclinical and early clinical trials due to the exponential nature of the data produced by the analysis. As a result, companies and regulatory agencies still favor clinical endpoints or surrogate endpoints at these early clinical stages, and nearly all biomarkers that are tested after treatment in these early clinical studies are used to correlate with the probability or magnitude of a drug response.

However, early clinical trials are ideal settings to explore biomarkers in relation to safety and efficacy for a number of reasons. First of all, any biomarkers that show a correlation with safety or efficacy could eventually serve as surrogate endpoints, which can then be used for future large-scale clinical trials to speed up the trials and reduce the costs. Secondly, biomarker testing, which would otherwise be deemed as unnecessary after demonstration of activity, could also facilitate future large clinical trials. For instance, somatic mutations in exon 19 of EGFR observed after demonstration of efficacy of EGFR tyrosine kinase inhibitors (e.g. gefitinib and erlotinib) [64, 65] could help identify a population that is most likely to respond. Furthermore, even biomarker testing in failed clinical trials could perhaps provide an explanation for drug failure and help to better design future drugs. Finally, a genetic predisposition such as a single nucleotide polymorphism may not be considered as a biomarker by Zolg and Langen [9], but it certainly offers an opportunity to identify populations more likely to benefit from treatment with a particular drug or more prone to suffer from serious side-effects. Ideally, it is recognized that such exploratory clinical studies may be conducted independently to identify and validate biomarkers, in particular, if tool compounds are available.

Later Clinical Stages

Expanded controlled and uncontrolled Phase 3 trials are required to collect the additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug for specific and expanded indications and provide an adequate basis for extrapolating the results to the general population. At this stage, incorporation of validated surrogate biomarkers for the stratification and monitoring of patients may contribute to the most significant cost-savings in drug development. In addition, the cost to incorporate transcriptomics, as done presently, would be prohibitive at this point. However, all surrogate endpoints will require clinical validation, preferably from prospective randomized clinical trials incorporating untreated control groups, and regulatory approval. Approval of a biomarker as a surrogate endpoint is typically derived from a large database across multiple phase 3 clinical trials and validated by large scale epidemiology studies. A common characteristic of all surrogate endpoints is their broad and robust applications. For instance, both the American Cancer Society and the American Urological Association recommend annual testing of serum prostate-specific antigen (PSA) for all men over age 50 and for those at a higher risk over age 40 such as African Americans and men with a strong family history of prostate cancer [66]. PSA can not only be used for early detection (screening) and prognosis of human prostate cancer, but it can also be employed to monitor real-time responses to various therapeutic interventions [67-69]. In fact, the FDA has approved several PSA tests for monitoring

recurrence of prostate cancer in men being treated for the disease since 1985. Furthermore, it can serve as an intermediate surrogate indicative of the effectiveness of treatments in chemopreventive trials [70], which would otherwise take much longer and cost much more to complete. Examples of other such surrogate biomarkers that have been used successfully to expedite clinical trials in their respective disease areas include bone mineral density or bone turnover markers in osteoporosis [71-75], blood glucose or glycosylated hemoglobin levels in diabetes [76-81], cholesterol levels in cardiovascular diseases [51, 52, 82-85], CD4 counts in HIV infections [54, 55, 86, 87].

Marketing Stage

Biomarkers can be very valuable in the diagnosis and prognosis of a medical condition for which a specific therapy is intended. The American Society of Clinical Oncology provides clinical practice guidelines for the care of patients outside of clinical trials which include the use of tumor marker tests in the prevention, screening, treatment, and surveillance of breast and colorectal cancers in [88]. For instance, HER2/neu overexpression and hormone receptor status (i.e. estrogen receptors and progesterone receptors) are evaluated on every primary breast cancer either at the time of diagnosis or at the time of recurrence to identify patients most likely to benefit from monoclonal (i.e. trastuzumab) or endocrine (e.g. tamoxifen) forms of adjuvant therapy and therapy for recurrent or metastatic disease. More

importantly, fully developed tests for clinically validated biomarkers will help doctors to identify those patients with the maximum benefit-risk ratios and to monitor their responses over the course of the treatment. For instance, a simple measurement of cholesterol level in the blood enables doctors to identify those at risk for cardiovascular diseases and to prescribe cholesterol-lowering drugs. A series of subsequent measurements of the cholesterol levels will allow doctors to monitor the effectiveness of the prescribed drug and, if necessary, change the course of treatment. In the case of HIV patients undergoing cocktail therapy, the viral load test and viral mutation test will allow physicians to monitor potential drug resistance to therapy and adjust the "cocktail" accordingly [87].

It should be pointed out that tests for biomarkers critical for patient management require regulatory approval and development of the tests often involve partnership with specialized diagnostic companies. Many pharmaceutical companies still face the challenge of incorporating development of biomarkers into the drug development paradigm (Figure 1). In addition, the number of tests with regulatory approval is also very low and insufficient to meet the demand for such tests.

BIOMARKER ISSUES AND FUTURE PERSPECTIVES

The potential impact of biomarkers in drug development is enormous. Biomarkers will play an increasingly important role in all phases of drug development from identification of "druggable" targets in discovery, to earlier and robust measurements of drug safety and efficacy in preclinical studies through clinical trials (Table 3) [89]. However, development of biomarkers carries some inherent challenges. For instance, use of biomarkers to stratify patient populations according to the likelihood of a positive drug response or occurrence of unwanted side effects should expedite clinical trials by eliminating those patients with undesirable profiles. But it could also potentially reduce the accessibility of the drug to those patients with unmet medical needs that may have benefited from the treatment even in the absence of the "desired" biomarker profile. The invisible hand behind the drug development economy will only reward such a "personalized treatment" approach that is affordable and acceptable to the society. It also remains unclear how biomarker data will benefit regulatory filing for a new drug. In fact, pharmaceutical companies may even be reluctant to collect biomarker data, which could lead to delays in regulatory decisions or restrictions in labeling [90]. In addition, since identification and validation of biomarkers along with development and validation of biomarker assays is a lengthy and costly process (Figure 1); Table 4) [63, 89, 91], development of biomarkers by one company could offer shortcuts to its competitors. Interested parties will have to

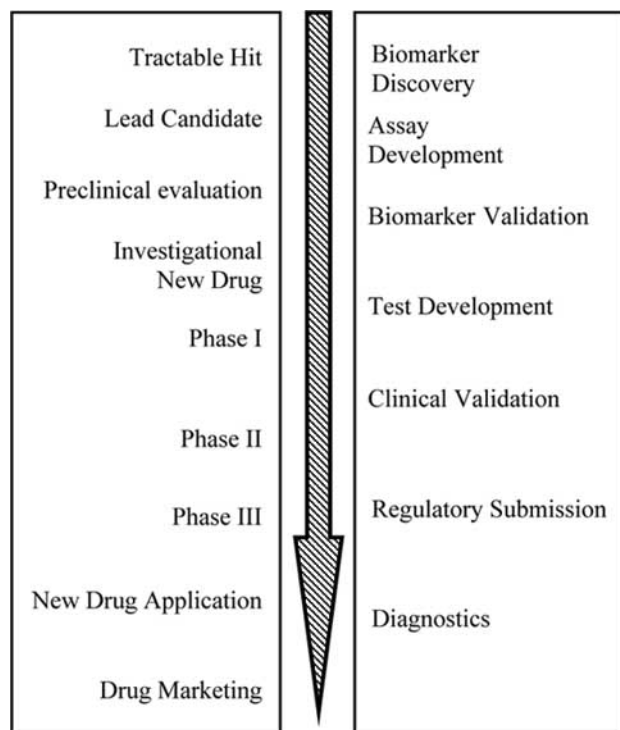


Figure 1. Parallel Paths of Drug Development (left column) and Biomarker Development. (right column).

Table 4. Biomarkers development issues.

Stages	Major Issues
Biomarker Discovery	<ul style="list-style-type: none"> • Molecular profiling – experimental design and data interpretation • Imaging biology – throughput and cost • Biomarker validation – epidemiology studies
Prototype Development	<ul style="list-style-type: none"> • Assay development – platforms, reagents, protocols • Analytic validation – sensitivity/specificity and alpha-site testing
Biomarker Test Development	<ul style="list-style-type: none"> • Production – process standardization and quality assurance • Clinical validation – clinical trials and beta-site testing • Marketing – registration

establish some “rules” of the game that provide incentives for such biomarker discovery, and may need to construct a consortium of biomarkers across a broad alliance with industry, academic institutions and government agencies. In addition, even generally accepted surrogate endpoints are unlikely to capture all the therapeutic benefits and potential adverse effects a drug will have in a diverse patient population [63]. For instance, pravastatin therapy lowers cholesterol levels, but measurements of cholesterol levels alone would underestimate its therapeutic benefit in reducing myocardial infarction risk [92]. Accordingly, combinations of biomarkers, such as patterns or signatures of gene expression as determined by transcriptome analysis, will probably be needed to provide a more complete characterization of the spectrum of pharmacologic responses. Realization of the full potential of transcriptome analysis, however, requires improved standardization of platforms and protocols, expanded functional annotations for many genes, and a better understanding of the pathophysiological or pharmacological relevance of the observed transcriptional alterations in the context of disease progression or therapeutic intervention [93]. In the future, pharmacogenomic approaches, including those based on differential expression of gene arrays, in combination with other biomarker discovery technologies such as proteomics and other “omics” (see article by Bilello in this issue), will provide panels of relevant biomarkers that can be expected to transform the drug development process. The general acceptance of clinically validated biomarkers as surrogates of disease management and therapeutic benefit by the regulatory authorities will be the most critical step leading towards this transformation.

REFERENCES

- [1] Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) *Science*, **270**, 467-470.
- [2] Lockhart, D.J., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. and Brown, E.L. (1996) *Nature Biotechnol.*, **14**, 1675-1680.
- [3] DeRisi, J., Penland, L., Brown, P.O., Bittner, M.L., Meltzer, P.S., Ray, M., Chen, Y., Su, Y.A. and Trent, J.M. (1996) *Nat. Genet.*, **14**, 457-460.
- [4] Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O. and Davis, R.W. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 10614-10619.
- [5] Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., Gaasterland, T., Glenisson, P., Holstege, F.C., Kim, I.F., Markowitz, V., Matese, J.C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J. and Vingron, M. (2001) *Nat. Genet.*, **29**, 365-371.
- [6] DiMasi, J.A., Hansen, R.W. and Grabowski, H.G. (2003) *J. Health Econ.*, **22**, 151-185.
- [7] Biomarkers Definitions Working Group (2001) *Clin. Pharmacol. Ther.*, **69**, 89-95.
- [8] Congress, O. h. f. Senate Bill 830 Food and Drug Administration Modernization Act of 1997. **10** (1997).
- [9] Zolg J.W. and Langen, H. (2004) *Mol. Cell Proteomics*, **3**, 345-354.
- [10] The Pink Sheet, Nov. 10 (2003).
- [11] Bargmann, C.I., Hung, M.C. and Weinberg, R.A. (1986) *Nature*, **319**, 226-230.
- [12] Weiner, D.B., Liu, J., Cohen, J.A., Williams, W.V. and Greene, M.A. (1989) *Nature*, **339**, 230-231.
- [13] Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and McGuire, W.L. (1989) *Science*, **244**, 707-712.
- [14] Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J.M., Paton, V., Shak, S., Lieberman, G. and Slamon, D. J. (1999) *J. Clin. Oncol.*, **17**, 2639-2648.
- [15] Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J. and Norton, L. (2001) *N. Engl. J. Med.*, **344**, 783-792.
- [16] Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., Shak, S., Stewart, S.J. and Press M. (2002) *J. Clin. Oncol.*, **20**, 719-726.
- [17] Versola, M., Burris, H.A., Jones, S., Wilding, G., Taylor, C., Pandite, L., Smith, D.A., Stead, A. and Spector, N. L. (2004) *J. Clin. Oncol.*, **22**(14S), 3047.
- [18] Allen, L.F., Eiseman, I.A., Fry, D.W. and Lenehan, P.F. (2003) *Semin. Oncol.*, **30**(S16), 65-78.
- [19] Drews, J. and Ryser, S. (1997) *Nat. Biotechnol.*, **15**, 1318-1319.
- [20] Hopkins A.L. and Groom C.R. (2002) *Nat. Rev. Drug Discov.*, **1**, 727-730.
- [21] Liotta, L. and Petricoin, E. (2000) *Nat. Rev. Genet.*, **1**, 48-56.
- [22] Ono, K., Tanaka, T., Tsunoda, T., Kitahara, O., Kihara, C., Okamoto, A., Ochiai, K., Takagi, T. and Nakamura, Y. (2000) *Cancer Res.*, **60**, 5007-5011.
- [23] Sallinen, S.L., Sallinen, P.K., Haapasalo, H.K., Helin, H.J., Helen, P.T., Schraml, P., Kallioniemi, O.P. and Kononen, J. (2000) *Cancer Res.*, **60**, 6617-6622.
- [24] Finlin, B.S., Gau, C.L., Murphy, G.A., Shao, H., Kimel, T., Seitz, R.S., Chiu, Y.F., Botstein, D., Brown, P.O., Der, C.J., Tamanoi, F., Andres, D.A. and Perou, C.M. (2001) *J. Biol. Chem.*, **276**, 42259-42267.
- [25] Gruvberger, S., Ringnér, M., Chen, Y., Panavally, S., Saal, L.H., Borg, A., Fernö, M., Peterson, C. and Meltzer, P.S. (2001) *Cancer Res.*, **61**, 5979-5984.
- [26] Sørlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lonning, P. and Borresen-Dale, A.L. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 10869-10874.
- [27] West, M., Blanchette, C., Dressman, H., Huang, E., Ishida, S., Spang, R., Zuzan, H., Olson, J.A. Jr., Marks, J.R. and Nevins, J.R. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 11462-11467.
- [28] van 't Veer L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., Schreiber G.J., Kerkhoven, R.M., Roberts, C., Linsley, P.S., Bernards, R. and Friend, S.H. (2002) *Nature*, **415**, 530-536.
- [29] van de Vijver, M.J., He, Y.D., van't Veer, L.J., Dai, H., Hart, A.A., Voskuil, D.W., Schreiber, G.J., Peterse, J.L., Roberts, C.,

- Marton, M.J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E.T., Friend, S.H. and Bernards, R. (2002) *N. Engl. J. Med.*, **347**, 1999-2009.
- [30] Sotiriou, C., Neo, S.Y., McShane, L.M., Korn, E.L., Long, P.M., Jazaeri, A., Martiat, P., Fox, S.B., Harris, A.L. and Liu, E.T. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 10393-10398.
- [31] Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A. and Chinnaiyan, A.M. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 9309-9314.
- [32] Afshari, C.A., Nuwaysir, E.F. and Barrett, J.C. (1999) *Cancer Res.*, **59**, 4759-4760.
- [33] Ulrich, R. and Friend, S.H. (2002) *Nat. Rev. Drug Discov.*, **1**, 84-88.
- [34] Storck, T., von Brevern, M.C., Behrens, C.K., Scheel, J. and Bach, A. (2002) *Curr. Opin. Drug Discov. Devel.*, **5**, 90-97.
- [35] Guerreiro, N., Staedtler, F., Grenet, O., Kehren, J. and Chibout, S.D. (2003) *Toxicol. Pathol.*, **31**, 471-479.
- [36] Lord, P.G. (2004) *Toxicol. Lett.*, **149**, 371-375.
- [37] Aardema, M.J. and MacGregor, J.T. (2002) *Mutat. Res.*, **499**, 13-25.
- [38] Kennedy, T. (1997) *Drug Discov. Today*, **2**, 436-444.
- [39] Tomaszewski, J.E. (2004) *Eur. J. Cancer*, **40**, 907-913.
- [40] Bulera, S.J., Eddy, S.M., Ferguson, E., Jatko, T.A., Reindel, J.F., Bleavins, M.R. and DeLa Iglesia, F.A. (2001) *Heptology*, **33**, 1239-1258.
- [41] Gerhold, D., Lu, M., Xu, J., Austin, C., Caskey, C.T. and Rushmore, T. (2001) *Physiol. Genomics*, **5**, 161-170.
- [42] Certa, U., Seiler, M., Padovan, E. and Spagnoli, G.C. (2001) *Br. J. Cancer*, **85**, 107-114.
- [43] Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T.G., Andrews, D.T., Scudiero, D.A., Eisen, M.B., Sausville, E.A., Pommier, Y., Botstein, D., Brown, P.O. and Weinstein, J.N. (2000) *Nat. Genet.*, **24**, 236-244.
- [44] Barker, A.J., Gibson, K.H., Grundy, W., Godfrey, A.A., Barlow, J.J., Healy, M.P., Woodburn, J.R., Ashton, S.E., Curry, B.J., Scarlett, L., Henthorn, L. and Richards, L. (2001) *Bioorg. Med. Chem. Lett.*, **11**, 1911-1914.
- [45] Wakeling, A.E., Guy, S.P., Woodburn, J.R., Ashton, S.E., Curry, B.J., Barker, A.J. and Gibson, K.H. (2002) *Cancer Res.*, **62**, 5749-5754.
- [46] Kris, M.G., Natale, R.B., Herbst, R.S., Lynch, T.J. Jr., Prager, D., Belani, C.P., Schiller, J.H., Kelly, K., Spiridonidis, H., Sandler, A., Albain, K.S., Cella, D., Wolf, M.K., Averbuch, S.D., Ochs, J.J. and Kay, A.C. (2003) *JAMA*, **290**, 2149-2158.
- [47] Miller, V.A., Kris, M.G., Shah, N., Patel, J., Azzoli, C., Gomez, J., Krug, L.M., Pao, W., Rizvi, N., Pizzo, B., Tyson, L., Venkatraman, E., Ben-Porat, L., Memoli, N., Zakowski, M., Rusch, V. and Heelan, R.T. (2004) *J. Clin. Oncol.*, **22**, 1103-1109.
- [48] Parra, H.S., Cavina, R., Latteri, F., Zucali, P.A., Campagnoli, E., Morengi, E., Grimaldi, G.C., Roncalli, M. and Santoro, A. (2004) *Br. J. Cancer*, **91**, 208-212.
- [49] Jordan, M.A. (2002) *Curr. Med. Chem. Anti-Canc. Agents*, **2**, 1-17.
- [50] Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M., Cao, Y., Shujath, J., Gawlak, S., Eveleigh, D., Rowley, B., Liu, L., Adhane, L., Lynch, M., Auclair, D., Taylor, I., Gedrich, R., Voznesensky, A., Riedl, B., Post, L.E., Bollag, G. and Trail, P.A. (2004) *Cancer Res.*, **64**, 7099-7109.
- [51] Scandinavian Simvastatin Survival Study Group. (1994) *Lancet*, **344**, 1383-1389.
- [52] Heart Protection Study Collaborative Group. (2002) *Lancet*, **360**, 7-22.
- [53] Ridker, P.M. (2002) *Circulation*, **105**, 2583-2585.
- [54] Hughes, M.D., Daniels, M.J., Fischl, M.A., Kim, S. and Schooley, R.T. (1998) *AIDS*, **12**, 1823-1832.
- [55] Gallant, J.E. (2002) *J. Clin. Virol.*, **25**, 317-333.
- [56] De Franco, A.C. and Nissen, S.E. (2001) *Am. J. Cardiol.*, **88**, 7M-20M.
- [57] Nissen, S. (2002) *Am. J. Cardiol.*, **89**, 24B-31B.
- [58] Nieman, K., Cademartiri, F., Lemos, P.A., Raaijmakers, R., Pattynama, P.M. and de Feyter, P.J. (2002) *Circulation*, **106**, 2051-2054.
- [59] Jacobs, L.D., Beck, R.W., Simon, J.H., Kinkel, R.P., Brownscheidle, C.M., Murray, T.J., Simonian, N.A., Siasor, P.J. and Sandroock, A.W. (2000) *N. Engl. J. Med.*, **343**, 898-904.
- [60] Therasse, P., Arbuck, S.G., Eisenhauer, E.A., Wanders, J., Kaplan, R.S., Rubinstein, L., Verweij, J., Van Glabbeke, M., van Oosterom, A.T., Christian, M.C. and Gwyther, S.G. (2000) *J. Natl. Cancer Inst.*, **92**, 205-216.
- [61] Wise, R.G., Rogers, R., Painter, D., Bantick, S., Ploghaus, A., Williams, P., Rapeport, G. and Tracey, I. (2002) *Neuroimage*, **16**, 999-1014.
- [62] Borsook, D. and Bercerra, L. (2002) *Curr. Opin. Investig. Drugs*, **3**, 1342-1347.
- [63] Frank, R. and Hargreaves, R. (2003) *Nat. Rev. Drug Discov.*, **2**, 566-580.
- [64] Paez, J.G., Janne, P.A., Lee, J.C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F.J., Lindeman, N., Boggon, T.J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M.J., Sellers, W.R., Johnson, B.E. and Meyerson, M. (2004) *Science*, **304**, 1497-1500.
- [65] Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G., Louis, D.N., Christiani, D.C., Settleman, J. and Haber, D.A. (2004) *N. Engl. J. Med.*, **350**, 2129-2139.
- [66] Smith, R.A., Cokkinides, V. and Eyre, H.J. (2004) *CA Cancer J. Clin.*, **54**, 41-52.
- [67] Catalona, W.J., Partin, A.W., Slawin, K.M., Brawer, M.K., Flanigan, R.C., Patel, A., Richie, J.P., deKernion, J.B., Walsh, P.C., Scardino, P.T., Lange, P.H., Subong, E.N., Parson, R.E., Gasior, G.H., Loveland, K.G. and Southwick, P.C. (1998) *JAMA*, **279**, 1542-1547.
- [68] Lieberman, R. (2004) *Am. J. Ther.*, **11**, 501-506.
- [69] Walczaka, J.R. and Carducci M.A. (2003) *Urology*, **62** (suppl 1), 141-146.
- [70] Trock, B.J. (2001) *Urology*, **57**(Suppl 1), 241-247.
- [71] Marshall, D., Johnell, O. and Wedel, H. (1996) *BMJ*, **312**, 1254-1259.
- [72] Karpf, D.B., Shapiro, D.R., Seeman, E., Ensrud, K.E., Johnston, C.C. Jr., Adami, S., Harris, S.T., Santora, A.C. 2nd., Hirsch, L.J., Oppenheimer, L. and Thompson, D. (1997) *JAMA*, **277**, 1159-1164.
- [73] Johnell, O., Scheele, W.H., Lu, Y., Reginster, J.Y., Need, A.G. and Seeman, E. (2002) *J. Clin. Endocrinol. Metab.*, **87**, 985-992.
- [74] Bone, H.G., Hosking, D., Devogelaer, J.P., Tucci, J.R., Emkey, R.D., Tonino, R.P., Rodriguez-Portales, J.A., Downs, R.W., Gupta, J., Santora, A.C. and Liberman, U.A. (2004) *N. Engl. J. Med.*, **350**, 1189-1199.
- [75] Meunier, P.J., Roux, C., Seeman, E., Ortolani, S., Badurski, J.E., Spector, T.D., Cannata, J., Balogh, A., Lemmel, E.M., Pors-Nielsen, S., Rizzoli, R., Genant, H.K. and Reginster, J.Y. (2004) *N. Engl. J. Med.*, **350**, 459-468.
- [76] Wang, P.H., Lau, J. and Chalmers, T.C. (1993) *Lancet*, **341**, 1306-1309.
- [77] Campbell, I.W. and Howlett, H.C. (1995) *Diabetes Metab. Rev.*, **11**, S57-S62.
- [78] Groeneveld, Y., Petri, H., Hermans, J. and Springer, M.P. (1999) *Diabet. Med.*, **16**, 2-13.
- [79] Coutinho, M., Gerstein, H.C., Wang, Y. and Yusuf, S. (1999) *Diabetes Care*, **22**, 233-240.
- [80] Marshall, J., Jennings, P., Scott, A., Fluck, R.J. and McIntyre, C.W. (2003) *Kidney Int.*, **64**, 1480-1486.
- [81] Peters, A.L., Davidson, M.B., Schriger, D.L. and Hasselblad, V. (1996) *JAMA*, **276**, 1246-1252.
- [82] National Cholesterol Education Program (NCEP) Expert Panel. (1993) *JAMA*, **269**, 3015-3023.
- [83] Joint Task Force of European and Other Societies on Coronary Prevention. (1998) *Eur. Heart J.*, **19**, 1434-1503.
- [84] Gould, A.L., Rossouw, J.E., Santanello, N.C., Heyse, J.F., Furberg, C.D. (1998) *Circulation*, **97**, 946-952.
- [85] Olsson, A.G. (2001) *Am. J. Cardiol.*, **87**, 33B-36B.
- [86] Gilbert, P.B., DeGruttola, V.G., Hudgens, M.G., Self, S.G., Hammer, S.M. and Corey, L. (2003) *J. Infect. Dis.*, **188**, 179-193.
- [87] Patarca, R., Isava, A., Campo, R., Rodriguez, N.J., Nunez, E., Alter, M., Marchette, M., Sanabria, M.M., Mitchell, C., Rivera, D., Scott, G., Jayaweera, D., Moreno, J., Boulanger, C., Kolber, M., Mask, C.W., Sierra, E.M., Vallejo, R., Page, J.B., Klimas, N.G.

- and Fletcher, M.A. (2003) *J. Environ. Pathol. Toxicol. Oncol.*, 22, 201-234.
- [88] Bast, R.C. Jr., Ravdin, P., Hayes, D.F., Bates, S., Fritsche, H. Jr., Jessup, J.M., Kemeny, N., Locker, G.Y., Mennel, R.G. and Somerfield, M.R. (2001) *J. Clin. Oncol.*, 19, 1865-1878.
- [89] Colburn, W.A. (2003) *J. Clin. Pharmacol.*, 43, 329-341.
- [90] Rolan, P., Atkinson, A.J. Jr. and Lesko, L.J. (2003) *Clin. Pharmacol. Ther.*, 73, 284-291.
- [91] Naylor, S. (2003) *Expert Rev. Mol. Diagn.*, 3, 525-529.
- [92] West of Scotland Coronary Prevention Study Group (1998) *Circulation*, 97, 1440-1445.
- [93] Bailey, W.J. and Ulrich, R. (2004) *Expert Opin. Drug Saf.*, 3, 137-151.