

## REVIEW

# Revitalizing Personalized Medicine: Respecting Biomolecular Complexities Beyond Gene Expression

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Despite recent advancements in “omic” technologies, personalized medicine has not realized its fullest potential due to isolated and incomplete application of gene expression tools. In many instances, pharmacogenomics is being interchangeably used for personalized medicine, when actually it is one of the many facets of personalized medicine. Herein, we highlight key issues that are hampering the advancement of personalized medicine and highlight emerging predictive tools that can serve as a decision support mechanism for physicians to personalize treatments.

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### CURRENT STATUS OF PERSONALIZED MEDICINE

The healthcare system has witnessed tremendous progress during the last century on several fronts. Survival rates have improved for many diseases. Several infectious diseases, such as polio and smallpox, have been practically eradicated among humans. Despite these advances, other conditions such as cancer, coronary artery diseases, HIV, and many more still pose a great challenge to healthcare providers and researchers alike. The human genome project identified and mapped ~23,000 genes.<sup>1</sup> A complete working draft of the human genome sequence was made freely available. This led the way to new advancements in the areas of molecular genetics, life sciences, biotechnology, and molecular biology. Despite the fact that 99.9% of human DNA sequences are identical, the 0.1% variation cascades into huge differences in disease susceptibility, disease progression, and response to intervention among individuals.<sup>2</sup> Since the human genome project, efforts have been underway to adopt genomic medicine in order to: (i) identify specific genes that are responsible for common hereditary diseases and aberrations in major pathways leading to illness, (ii) elucidate the underlying molecular mechanism of disease, (iii) identify potential therapeutic targets, (iv) design small-molecule drugs to intervene in the disease processes, (v) predict responses to treatment, and (vi) predict responses to drug intervention.

Personalized medicine is critically important and hence is increasingly favored in many areas of medicine, especially in oncology due to the complexities of the disease and lethality of the chemotherapeutics. A meta-analysis of 39 prospective studies from the US hospitals estimated the overall incidence of serious adverse drug reactions at a rate of 6.7%.<sup>3</sup> In this study, more than 2.2 million hospitalized patients had serious adverse drug reactions and ~106,000 patients had fatal adverse drug reactions, making it between the fourth and sixth leading cause of death in the United States. The cost of drug-related morbidity and mortality was estimated to be more than US\$177 billion in the year 2000.<sup>4</sup> In addition to these acute adverse drug reactions, patients receiving incompatible and inordinate treatments can suffer several

long-term medical and socioeconomic complications. For example, relapsed cancer, secondary neoplasms, heart disease, and many other chronic medical conditions are prevalent among long-term survivors of cancer.

Personalized treatment, when applied in clinical settings, helps to answer two important questions: (i) for a given individual, what drug or combination of drugs should be given to treat a specific disease condition? And (ii) How much and when should the drug(s) be administered? Pharmacogenomics, a field that has evolved in the last decade, has been highly recommended for several disease conditions toward predicting the response for a planned treatment protocol on an individual basis and has been put into practice in some cases. Pharmacogenomics has shown great promise in predicting the treatment response for a given patient and has demonstrated the ability to alleviate much of the morbidity that can be associated with treatment,<sup>5,6</sup> making it an excellent tool to address the first of the two questions above. However, because the purview of pharmacogenomics is limited to genotypic variation, it has limited scope to comprehensively answer the second question, which is at least as important to personalized treatment.

In addition to genetic variation, several other nongenetic molecular mechanisms interface within the human body. The manifestation of a specific gene sequence into a final disease outcome, with or without drug intervention, proceeds at various levels. First, the genes are transcribed and translated into proteins which act as enzymes in numerous metabolic reactions. Some proteins act as receptors and transporters to interface with the extracellular environment. For each gene encoding a specific protein, variant alleles may exist. This results in a certain pattern of endogenous metabolic fluxes and metabolic products. If a specific gene is implicated in drug disposition, the gene expression also affects the distribution, metabolism, and elimination of the compound.<sup>7</sup> The resultant phenotypes at the bio-atomic or -molecular level then exert phenotypic changes at the cellular, tissue, and organ level through their influence on the disease and response pathways. Variations/aberrations, not only in gene sequence and expression, but in any of the steps mentioned

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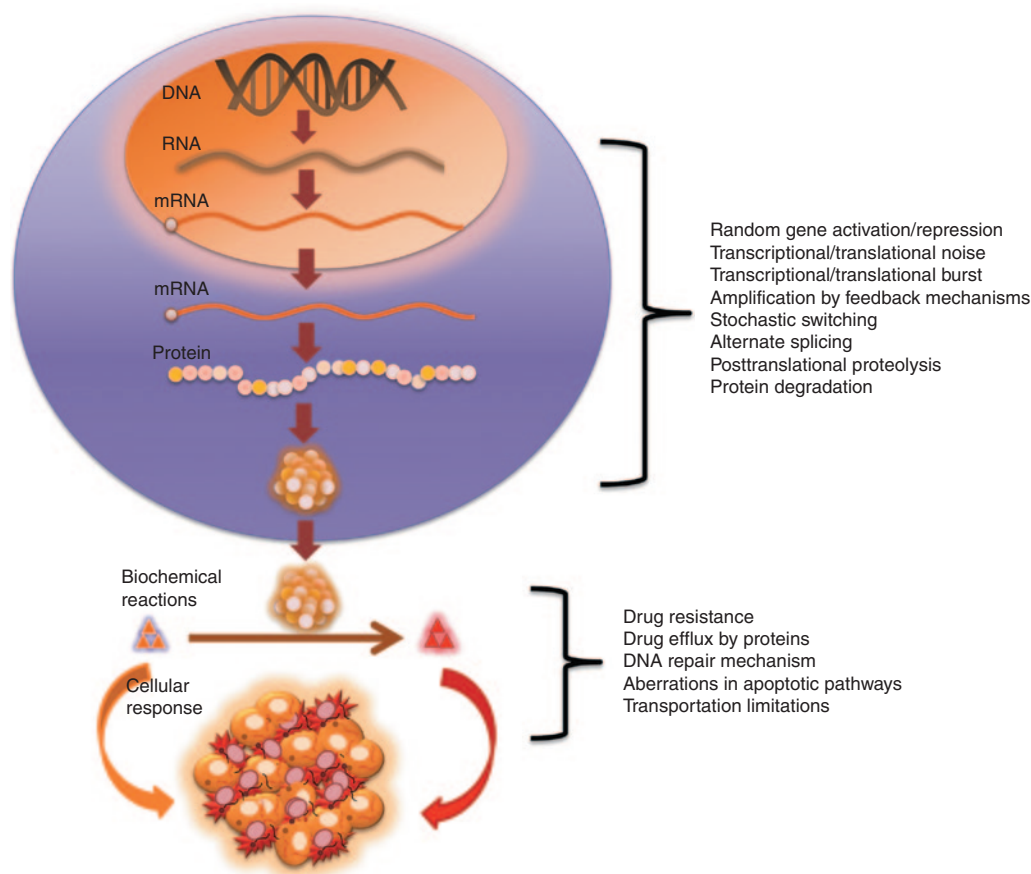
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above, will result in an unexpected outcome at an organismal level. **Figure 1** enumerates the cascade of events and validated processes contributing to the variations in these steps. The clinical implication of these events is the possibility of observing a subgroup of patients with same genotype but unique proteome, metabolome, and cellular responses, which results in completely different treatment outcomes for each individual patient. The key factors, besides gene expression, responsible for such consequences include—but are not limited to—epigenetic factors, nonheritable functionally induced (extragenetic) factors, stochasticity in biochemical reactions, interactions in signal transduction and metabolic networks, environment, nutrition/lifestyle, organ failure (renal, hepatic), coadministered drugs, pregnancy, disease type, and microflora dynamics. Exclusive, isolated application of pharmacogenomics has therefore come under scrutiny by some in the literature and has resulted in a few new clinical guidelines that are broadly accepted for management of patients.<sup>8–10</sup>

The foregoing discussion substantiates the need for developing procedures to accurately measure and/or predict the phenotypic outcome for a specific pharmacogenomic variant. In addition, methodologies must also be developed to determine optimal dosing of drug to realize a specific phenotypic outcome. Traditionally, pharmaceutical companies prescribe the “optimal” dosing information based on clinical

trials conducted at a population level of the general public and patients. Thus, it is primarily a statistical consolidation imposed on an individual patient. The adoption of standard-dose-for-all approach from drug labels and trial-and-error approach to titrate the patient to maximum tolerated dose has resulted in severe toxicity in some patients and insufficient treatment in others. The so-called evidence-based medicine and the adoption of treatment standards based on large epidemiological studies or randomized controlled trials have significantly hampered the efforts to truly personalize the medical practice.<sup>11</sup> A paradigm shift from evidence-based medicine to mechanism-based medicine will ensure that each patient is treated according to his/her own mechanism and thus should be the impetus for expanding implementation and utilization of personalized medicine.

In this work, we highlight some of the limitations associated with the isolated application of pharmacogenomics to personalize treatment. Specific instances of biomolecular processes responsible for such outcomes are analyzed in detail with respect to each level of phenotype. Finally, emerging tools and methodologies to augment the potentials of pharmacogenomics for a comprehensive realization of personalized treatment are discussed. We first provide an introduction to the concept of pharmacogenomics, its prevalence, strengths, and limitations in personalizing treatment



**Figure 1** Manifestation of DNA sequence to molecular phenotypes and cellular responses. Each step in this process is confounded by several biochemical events that add dispersion and uncertainty to the subsequent steps. As such, it would be highly unlikely for there to exist a one-to-one relationship between a specific gene sequence and ultimate clinical outcome.

(see Pharmacogenomics and Gene Expression). We will then appraise the complexities in the molecular and cellular level phenotypic manifestation of gene sequences, highlighting potential processes responsible for this variation. The advantages of measuring/predicting cellular responses and challenges are also discussed (see Complexities in Predicting Molecular Phenotypes). Finally, we highlight some of the emerging technological and quantitative tools to extend the scope of personalized treatment beyond pharmacogenomics (see Future Directions and Emerging Technologies).

## PHARMACOGENOMICS AND GENE EXPRESSION

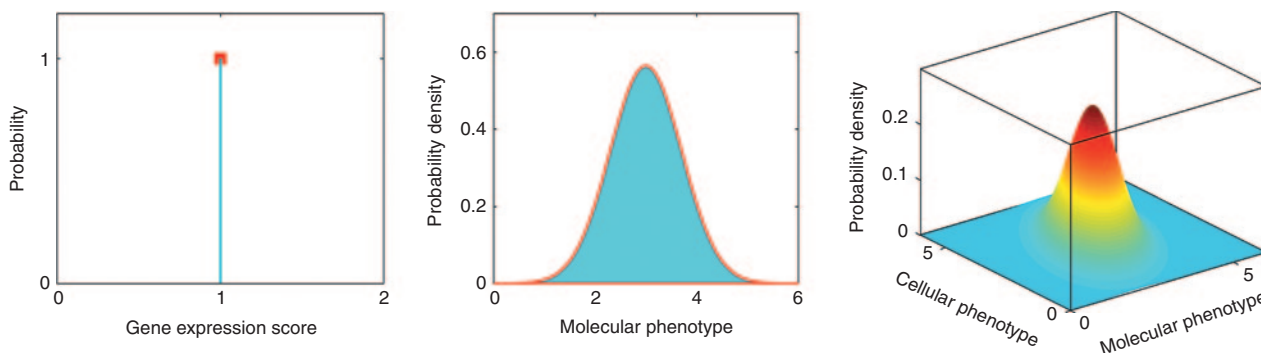
Gene sequencing and expression profiling are excellent tools for discerning variations in disease susceptibility, disease diagnosis and classification, and prognosis for a given treatment. Within the realm of personalized treatment, pharmacogenetics garners prime attention in utilizing gene sequencing tools for clinical translation. Pharmacogenetics aims to provide insight into the influence of genetic variants on the molecular biology of disease and response to drug intervention. The concept of genetic polymorphism in the human genome forms the core of pharmacogenomics. Common sources of genetic polymorphisms include single-nucleotide polymorphisms (SNPs), nucleotide repeats, deletions, insertions, and recombination. Pharmacogenomics possesses a great potential to propel the development of new therapeutic agents and/or administer existing drugs to a targeted subgroup of the patient population who display a specific genotypic trait. Gene expression analysis and pharmacogenomics are being considered as companion diagnostic tools (tests recommended when prescribing a specific medication) in several cases.<sup>10</sup>

The first conceivable utility of gene expression variation in the disease cycle is the elucidation of disease susceptibility. A case-control study, conducted to demonstrate the association of *NRAMP1* gene and susceptibility to tuberculosis, estimated that the odds of developing tuberculosis is 4.07 among subjects who are heterozygous for two *NRAMP1* polymorphisms.<sup>12</sup> Genetic polymorphism has also been exploited in many studies to diagnose and classify the existing categories of many cancers. In a landmark work on molecular classification of cancer using gene expression, DNA microarray technique was utilized to distinguish between acute myeloid leukemia and acute lymphoblastic leukemia.<sup>13</sup> A study devoted to characterizing diffuse large B-cell lymphoma, a common form of non-Hodgkin's lymphoma, using microarray gene expression profile, revealed two molecularly distinct forms of diffuse large B-cell lymphoma.<sup>14</sup> These two new subtypes, germinal center B-like diffuse large B-cell lymphoma and activated B-like diffuse large B-cell lymphoma, are representative of different stages of B-cell differentiation and predict overall prognosis. These systematic and unbiased elucidations of disease subtypes, based on global gene expression profiles, not only assist clinicians in choosing appropriate treatment strategies that maximize efficacy but also minimize unwarranted side effects. Gene expression profiling also helps to direct the prediction of prognosis of the disease for a specific treatment regimen. One of the well-studied and clinically adopted examples of gene expression techniques is the demonstration of the relationship between

*HER-2/neu* gene and a wide variety of human cancers. Amplification of *HER-2/neu* gene or overexpression of HER-2/neu protein is observed in as many as 34% of the breast cancer patients.<sup>15</sup> In these patients, abnormalities in *HER-2/neu* gene and protein dictate relative sensitivity to chemotherapeutic drugs and resistance to tamoxifen. *HER-2/neu* gene amplification also predicts node status, tumor grade, overall survival, and time to relapse in breast cancer patients.

One of the classical examples of applications of pharmacogenetics is the incidence of genetic polymorphism in thiopurine *S*-methyltransferase (*TPMT*) gene among humans.<sup>16,17</sup> To date, it serves as a prototypic system for displaying the potential for the utilization of a pharmacogenomics-based approach to individualized drug dosing within clinical settings. *TPMT* is a cytosolic drug-metabolizing enzyme that plays a key role in the metabolism of purine antimetabolites such as 6-mercaptopurine (6-MP) and azathioprine.<sup>17</sup> Thiopurine is an immunosuppressant that is used to treat childhood acute lymphoblastic leukemia, inflammatory bowel diseases, autoimmune diseases, and immunosuppression following solid organ transplantation. *TPMT* catalyzes the *S*-methylation of thiopurines and promotes pathways leading to inactive metabolites of methylated mercaptopurines. Hence, *TPMT* activity level is inversely proportional to the amount of active cytotoxic metabolite, 6-thioguanine nucleotide (6-TGN), produced. Myelosuppression is the dose-limiting toxicity during thiopurine dosing. A total of 21 genetic polymorphisms have been identified in the *TPMT* gene which correlate with decreased *TPMT* activity levels and hence thiopurine-induced toxicity. *TPMT* \*1 is the "wild-type" allele; *TPMT* \*3A is the most common variant allele, found in ~5% of Caucasians, whereas *TPMT* \*3C is the most common variant allele found in East Asian population with a frequency of ~2%. *TPMT* \*3B is a rare allele. The presence of *TPMT*\*3A and \*3B results in extremely low or no *TPMT* enzyme activity, which leads to elevated levels of 6-TGN. If treated with a standard dose, patients who are homozygous for these alleles will encounter life-threatening myelosuppression and, in some cases, even secondary malignancies.<sup>16-18</sup> Thus, it was suggested that patients with low *TPMT* expression should be treated with substantially lower doses of thiopurines. On the other hand, many clinical studies concluded that efficacy will be compromised in patients with high *TPMT* activity who are treated with standard dosing schedules, and therefore, treatment with higher doses is recommended.<sup>18</sup>

In all the above examples, pharmacogenomics provides some vital information for predicting treatment outcome, but they are limited to population-level variations. In some sense, it is equivalent to segregating the population into a few response groups and disregarding intragroup variations. To be complete and effective, following the first step of "genetic personalization," individuals in each subgroup must be characterized based on the downstream response of that genotype. It is well-known that within a specific genotype, there is a distribution of phenotypes across the patient population (Figure 2). For example, in the case of the *TPMT* gene, although there are only five important genotypes in the human, there are as many enzyme activity levels (the manifestation of the *TPMT* gene) as there are patients. No two patients with same *TPMT* genotype will have an identical enzyme



**Figure 2** A hypothetical case for dispersion of biomolecular information from gene expression to molecular phenotype to cellular phenotype. For each specific gene variant (represented as gene score), there is a distribution of molecular phenotype among the patient population due to variations in random gene activation and repression, mRNA degradation, translational noise, alternate splicing, and protein degradation arising at the individual patient level. At the next level, for each value of molecular phenotype, there is a distribution of cellular response in the population due to protein phosphorylation, membrane drug efflux pumps, transportation limitations, and resistance mechanisms in apoptotic pathways. Eventually, two patients having the same gene variant might fall anywhere in the bivariate distribution in phenotype space. mRNA, messenger RNA.

activity level. In addition, only two-thirds of the total variance in TPMT activity is accounted through genotyping.<sup>19</sup> Recently, for warfarin, an important US Food and Drug Administration–approved drug to bear the pharmacogenomics information in the label, pharmacogenomic-guided treatment has been shown to have no significant difference in clinical outcome compared with the traditional treatment.<sup>20</sup> Other studies also point to declining scope for pharmacogenomics in guiding dose regimen, given the cost and effort involved.<sup>21</sup>

Complex diseases such as cancer, HIV infection, and many others are invariably treated with complex treatment regimens that often involve multiple drugs. When the drugs are influenced by more than one gene independently, pharmacogenomics-based approach alone may not be sufficient to predict the drug response. Consider a treatment involving a combination of three drugs and having genetic polymorphism in each of the drug-metabolizing enzymes with three different gene expression patterns (high, intermediate, and low). This will produce 27 ( $3^3$ ) unique gene expression profiles in a given patient population. If one or more drugs are also substrates for drug transporters, where genetic polymorphism and hence three different gene expressions are possible, the number surges to 81 ( $3^4$ ). When this is then translated into phenotypes and further into cellular response, it will produce a significant variation in the response. Besides clinically relevant SNPs and their influence over treatment outcomes, there are several other putative genetic polymorphisms that are yet to be characterized, which may play a significant role in determining the drug response.<sup>22</sup> In light of more than 150,000 validated SNPs, proteins, and interactions between them, this works out to be a mind-boggling diversity! However, when phenotypes are measured, which include the drug concentrations and/or cellular response, the characterization of patients may correlate more closely with clinical observations.

Additional complexity surfaces when the findings of the gene expression profiles are translated to the global population of different ethnic origin due to the inherent variation in disease susceptibility, risk, incidence, and response. For example, there is a significant variation observed in

vincristine-induced peripheral neuropathy among Caucasian and African-American patients undergoing treatment for precursor B-cell acute lymphoblastic leukemia.<sup>23</sup> Pharmacogenomic studies of vincristine-metabolizing enzyme CYP3A5 revealed the polymorphic expression between different races with ~70% of African-Americans expressing CYP3A5 compared with 20% of Caucasians.<sup>23</sup> Dose interruptions and average toxicity grades are significantly lower in African-American patients as a result of elevated metabolism and clearance of vincristine. If the dosing for African patients were to be determined based on studies on Caucasians, these patients will receive significantly lower exposure to vincristine.

Another important consideration to expand the scope of pharmacogenomics is related to its reliance on decision making under static conditions. Pharmacogenomics uses gene sequence and gene expression snapshots with the assumption that deterministic evolution of molecular events leads to predictable phenotypes. It considers each genetic variation as an independent causal factor for the observed response. It fails to take into account variation in transport limitations and the spatial heterogeneity of biochemical reactions. However, human physiology is a complex, dynamical system, and often, therapeutic responses are a manifestation of the interplay between many levels of physiological processes. Furthermore, human physiology is complicated by homeostatic feedback loops, molecular cross talk, and bypass mechanisms that can lead to unexpected therapeutic responses. These events might confound many physiological processes including, but not limited to, drug metabolism and disposition, drug transport, cellular targets and signaling pathways, and cellular response pathways (e.g., apoptosis, cell cycle control).<sup>24</sup> Thus, one must remain circumspect about the isolated assessment of pharmacogenomics (or any other upstream biomarker) as a stand-alone personalizing tool. The scope for controlled clinical trials, a gold standard accepted by the US Food and Drug Administration for validating efficacy and safety of a pharmacogenomic tests, to validate and adopt to the clinical practice are also limited as the resulting number of groups make such studies an expensive and time-consuming exercise. To this end, pharmacogenomics has not realized its

fullest potential in some of the drug–disease applications that were deemed as a classical case for pharmacogenomics-based personalization.<sup>8,9,25,26</sup> It therefore emerges that a more comprehensive approach to “personalization,” encompassing and integrating many dimensions and levels of human physiology, is needed to portray a complete picture of ongoing drug–disease dynamics.

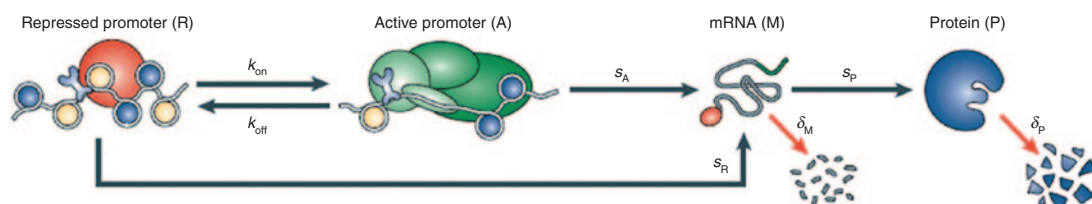
### COMPLEXITIES IN PREDICTING MOLECULAR PHENOTYPES

From the foregoing discussion, it is clear that gene sequencing and gene expression profiling play key roles in identifying patient subgroups, but individual patients are yet to be characterized on the phenotypic distribution. There are several levels of phenotypes, and a specific one depends on the objective at hand. In this work, we classify phenotypes into two broad categories: (i) molecular phenotypes, an immediate effect of a specific gene sequence, and (ii) cellular phenotypes, the influence of molecular phenotypes on various cell populations. We define molecular phenotypes as the biomolecular manifestation of a gene sequence, which encompasses proteins, enzymes (proteome), and metabolite concentrations (metabolic phenotype). If a specific drug distribution and metabolism is found to be modified due to the above factors, the resulting drug concentration at various parts of the body is also considered as the molecular phenotype (drug phenotype). Molecular phenotypes interact with various cellular populations as drug transporters, inhibitors, and signaling molecules to produce a cellular phenotype. Variations in phenotype arise due to numerous factors, including stochasticity in gene expression, transcriptional and translational noise, complexities in biochemical and signal transduction networks, nonlinearity in biochemical processes, and quasi-determinism in biological events.<sup>27,28</sup> This leads to a nonbijective relationship (a single gene producing more than one phenotypic trait and a specific phenotype resulting from the expression of several genes) between genotype and phenotype.

Stochasticity in gene expression is one of the important factors that contributes to phenotypic variations observed in isogenic cell populations.<sup>28</sup> These stochastic events are triggered by transcriptional and translational fluctuation which, in turn, arises due to several factors such as random activation/repression of promoter, degradation of transcriptional and protein products, transcriptional and translational burst, feedback loops, etc.<sup>28</sup> **Figure 3** demonstrates various molecular events during gene expression. Moreover, these gene

regulatory functions fluctuate dynamically, making static gene expression profiles untenable for personalized treatment. Besides quantitative variations in molecular contents, some of these mechanisms may lead to phenotypically distinct subpopulations. The majority of drugs are metabolized by more than one enzyme (e.g., 6-MP metabolized by TPMT, HGPRT (hypoxanthine-guanine phosphoribosyltransferase), and ITPA (inosine triphosphate pyrophosphatase) and transported/eliminated by several other proteins, where gene variations and some of the above-mentioned processes are inevitable. The end result is the formation of cell populations with uniquely different proteomes and metabolomes from the same genomes. The resulting phenotype is not comprehensible through simple deductive reasoning. As such, it is not uncommon for two genetically identical persons from similar backgrounds to show significantly different clinical phenotypes in response to drug intervention. For instance, inflammatory bowel disease patients with TPMT homozygous-w.t. and treated with 6-MP encountered completely different clinical outcome, both in terms of efficacy and toxicity.<sup>29</sup>

One of the main reasons for treatment failure and variability in cellular response, despite the drug being at a therapeutically effective concentration in the plasma, is the development of multidrug resistance to therapeutic medications mediated by drug transporters. This is especially true in critical diseases such as cancer and HIV infection. P-glycoprotein (P-gp), which is a member of the ATP-binding cassette (ABC) family of proteins, is located on the plasma membrane and serves as drug efflux pumps.<sup>7,30</sup> Important members of this family include ABCB1 and ABCG2 (BCRP). On the other hand, solute carrier super family of proteins, such as OATP family (e.g., OATP1B1, OATP1B3, and OATP2B1), act as uptake transporters.<sup>7</sup> Together, they have the ability to uptake/efflux structurally and functionally dissimilar cytotoxic agents, thereby modulating the intracellular drug concentration. These genes display genetic polymorphism in humans which has profound impact on pharmacokinetics and clinical responses to many drugs. Furthermore, stochastic and dynamic regulation of these genes result in heterogeneous population of three different types of cells—intrinsically resistant, acquired resistant, and sensitive cells. Intrinsically resistant cells acquire this phenotype due to a spontaneous mutation involving single or multiple random steps. Acquired resistant phenotypes are initially sensitive to drugs but eventually develop drug resistance, also through random processes. Recently, even sensitive cells have been shown to acquire the resistant phenotype from other resistant populations through the exchange of P-gp via microparticles



**Figure 3** Depiction of a single gene expression and regulation. Every step in this process is governed by stochastic biochemical events. The gene randomly transits between active and repressed promoter state and hence mRNA is produced in bursts. A fraction of mRNA is randomly degraded, and the rest is translated into protein. A fraction of protein also undergoes decay stochastically. Reprinted with permission from Macmillan Publishers: *Nature Reviews Genetics*.<sup>28</sup> copyright 2005. mRNA, messenger RNA.

and tunneling nanotubes.<sup>31</sup> The evolution of these cellular fractions during treatment has the greatest impact on clinical outcomes. Besides these genetic and epigenetic factors, drug resistance may also be conferred by microenvironment characterized by poor vasculature, spatial heterogeneity with regions of hypoxia and acidity, and transport limitations.<sup>32</sup> In these cases, despite abundant drug in plasma, the actual targeted site of action will lack the desired concentration of drug. Given these complex factors, sophisticated models incorporating probabilistic nature of these nongenetic, random events will greatly augment the predictions based on gene expression.

The final phase of drug–disease cycle involves the drug or its metabolites interfering with the normal functions of one or more cell types and producing either desired outcomes (efficacy) or undesired outcomes (side effects). Pharmacology literature refers to this as pharmacodynamics. The majority of these cell responses are governed by small molecules, which are the cumulative outcome of all of the processes outlined in the previous sections (gene sequence, drug dosing, and other biochemical processes). At the molecular level, activation or repression of gene and enzyme activity may not be translated into accumulation or depletion of its corresponding metabolites due to multiplicity of metabolic pathway network and robustness of metabolite profiles. In addition to these molecular contents, cellular functions are also affected by stochastic gene regulation. Due to the complex and interacting nature of these regulatory networks, gene products from a specific network can influence the production of proteins in some other unknown network. Ultimately, this results in the disruption of the normal cellular functions and produces unintended consequences.<sup>33</sup>

Developments in single-cell measurements and various lineage-tracing techniques have revealed a wealth of knowledge in attributing the sources of nongenetic cell-to-cell variations. Even genetically identical cells in the same environment have shown varied response and sensitivity to drugs.<sup>34</sup> Feinerman *et al.*<sup>35</sup> demonstrated how intraclonal differences in signaling protein levels in T cells produced distribution in response among individual cells, which ultimately leads to diverse biological functions and interferes with antigen discrimination during T-cell activation. Using 10,000 cells from each of 15 different cell lines, Gascoigne and Taylor<sup>36</sup> analyzed cellular response to three classes of antimetabolic drugs. These cells, besides inter–cell line variability, exhibited a significant intra–cell line variation. Indeed, these variations are not genetically predetermined but are driven by variations in the signaling network stability as even the sister cells were shown to have faced different fates. Spencer *et al.*<sup>37</sup> studied nongenetic cell-to-cell variability in response to TRAIL-induced (TNF-related apoptosis-inducing ligand) apoptosis. They have shown the existence of significant differences in timing and death probability in that some cells died within 45 min of exposure, whereas others needed as much as 8–12 h. Apart from genetic and epigenetic sources, stochastic fluctuations in biochemical reactions arising from low copy number, differences in cell cycle phase, and natural divergence of protein levels were cited as the determinant factors of time to death. Dynamic studies on negative feedback loops between tumor suppressor p53 and oncogene

Mdm2, with genetically identical cells in uniform environment subjected to gamma irradiation, revealed interesting features on cell-to-cell variability.<sup>38</sup> Significant cell-to-cell variations were observed in the amplitude of the oscillations that was attributed to the production rates of proteins. In addition, due to this variation in protein production, even sister cells lost correlation to each other within 11 h of cell division.

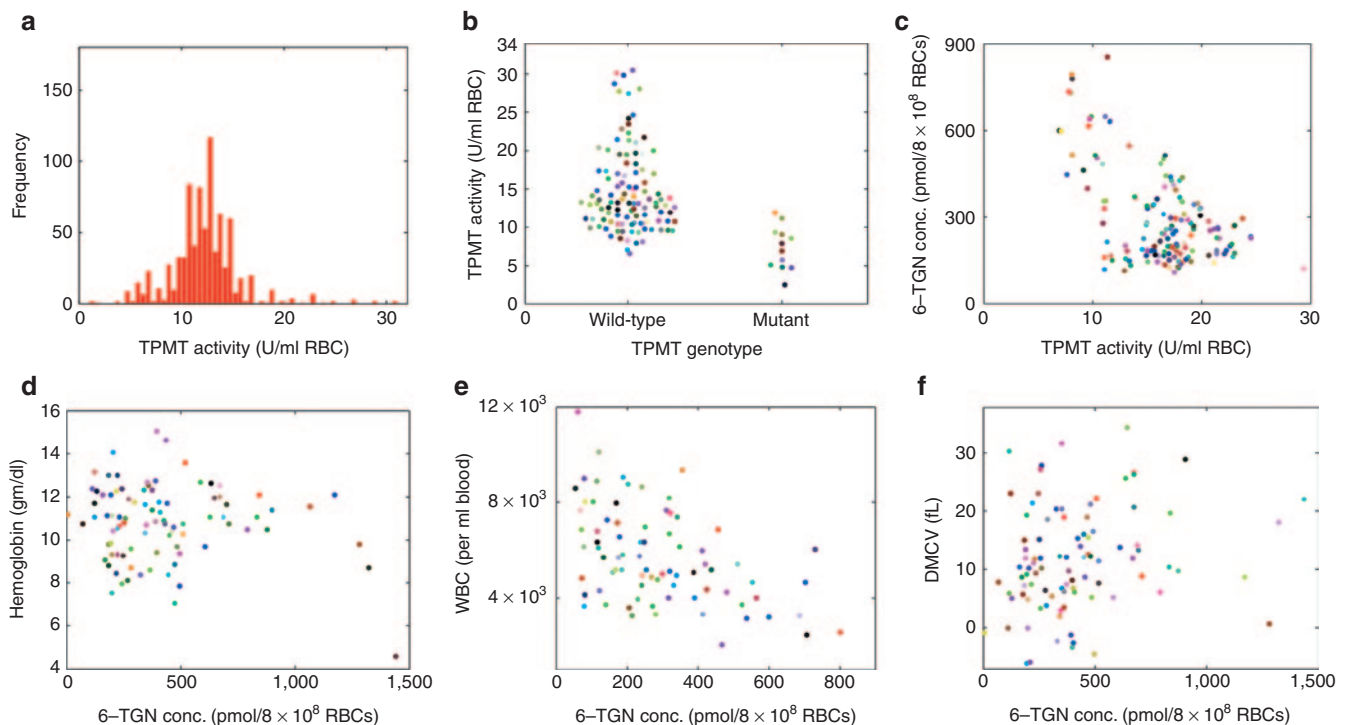
Cell-to-cell variability does not necessarily originate from stochastic fluctuations. Recent studies show that despite intrinsic noise in molecular network, phenotypic cell-to-cell variability is rendered by deterministic processes, often through uncharacterized molecular regulatory mechanisms.<sup>39</sup> Microenvironment plays a significant role in determining the ultimate outcome to any pathophysiological stimuli. *In vivo* experiments in mice seeded with invasive or proliferative melanoma cell types have shown that the melanoma cells experienced “transcriptional signature switching” resulting in heterogeneous distribution of both cell types.<sup>40</sup> In addition, proliferative cell types were predominant in the outer rim of the tumor confirming the role of microenvironment in regulating the switch. Studies have also illustrated the presence of small numbers of slow-cycling melanoma cells (JARID1B+), within the main population of aggressive cells, evading chemotherapy and resulting in the selection of JARID1B+ cells.<sup>41</sup> Interestingly, JARID1B expression is dynamically and temporarily regulated, thereby negating the utility of gene expression profiling. This has a profound impact on treatment planning; a snapshot of gene expression reveals a specific cell type but the emergence of heterogeneity renders the treatment regimen largely ineffective. Microenvironment also promotes genetic instability among cancer cells, specifically through deletion and transversions.<sup>42</sup> Despite the foregoing discussion on cell-to-cell variability, the inherent robustness in metabolic networks drive the physiological state to very few distinct modes, which results in multimodal distribution in response space.<sup>43</sup> Robust computational approaches are available to incorporate these multiple sources of stochasticity and heterogeneity, which enable prediction of population behavior.<sup>28,44</sup>

The relationship between cellular heterogeneity and unpredictable clinical response is less obvious but extremely critical. For example, in cancer cells, protein expression outliers allow some cells to fall outside the drug’s range of efficacy, enabling those cells to survive ongoing treatments.<sup>34</sup> Given sufficient time, these outliers will repopulate the full distribution of cells and render the treatment inefficient or eventually completely ineffective. The transcriptional switching in melanoma cells allows invasive cell types to escape the proliferation-targeted chemotherapy regimen.<sup>40</sup> When the invasive cell types switch to a proliferative mode, the tumor cells will regrow, thus leading to refractory melanoma. Chemotherapy also allows slow-cycling JARID1B+ melanoma cells in the heterogeneous population to thrive in cytotoxic environments. When the temporary expression of JARID1B is reverted, the melanoma will relapse. As individual patients produce different levels of proteome dispersion and cellular heterogeneity, which in itself is difficult to predict, the prediction of clinical response for these individuals is much more complex. More likely, these cellular phenotypes are further confounded by interplay of other unknown proteins and/or

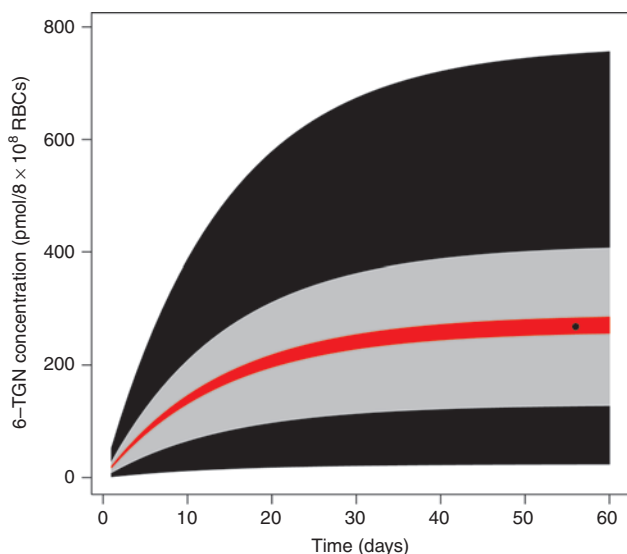
mechanisms. Hence, it is not so trivial to predict the cellular response intuitively or use simple correlations with gene expression alone. A more reliable quantitative prediction of these complex phenomena calls for a sophisticated modeling framework.

The literature is frequented with wide range of sources producing differing evidence for correlation between genotype and their corresponding molecular phenotype and/or clinical response.<sup>26,45,46</sup> Continuing with the *TPMT* case, population genetic studies have shown that the major gene locus which regulates *TPMT* activity accounts for only two-thirds of the total variance in the red blood cell (RBC) enzyme activity.<sup>19</sup> *TPMT* enzyme activity is affected by 6-MP, chronic diseases, and other coadministered drugs like diuretics, NSAIDs, and antihypertensives.<sup>47</sup> In addition, tissue-specific regulation of enzyme activity has also been reported in the literature.<sup>48</sup> 6-MP metabolism is not only affected by variations in *TPMT* activity but also by other nongenetic factors. Diet plays a significant role in determining the bioavailability of 6-MP. Coadministered drugs like allopurinol and methotrexate also affect the first-pass clearance and metabolism of 6-MP.<sup>49</sup> These factors indirectly affect the amount of 6-TGN produced. Hence, within a specific enzyme activity range, it is possible to observe as many 6-TGN concentrations as there are patients. In a study involving 170 inflammatory bowel disease patients, wide variation in *TPMT* enzyme activity, 6-TGN concentration, and treatment response were

observed.<sup>50</sup> Patients with *TPMT* heterozygous had enzyme activity of 5.1–13.7 U/ml with mean 6-TGN concentration of 253.5 pmol/8 × 10<sup>8</sup> RBCs (SD: 136.5) compared with homozygous-w.t. of >13.7 U/ml with 151 pmol/8 × 10<sup>8</sup> RBCs (SD: 84.7). The range for *TPMT* activity and high SD for 6-TGN concentration signifies the level of dispersion within the same genotype. In addition, there was no significant correlation existed between inflammatory bowel disease questionnaire score and 6-TGN concentration ( $r = -0.09$ ;  $P = 0.24$ ). Patient groups having similar 6-TGN concentrations resulted in two different clinical outcomes of active disease and clinical remission. In our own modeling study on 6-MP metabolism using nonparametric Bayesian approach (to be submitted for publication), 6-TGN concentration prediction based only on *TPMT* genotype resulted in wide 95% confidence region of 23–743 pmol/8 × 10<sup>8</sup> RBCs (black region in **Figure 5**). With the measurement of *TPMT* enzyme activity, the confidence region is narrower (gray region: 124–386 pmol/8 × 10<sup>8</sup> RBCs) as the variability in enzyme level is accounted. However, with the availability of 6-TGN measurement, the 95% confidence region is much tighter (red region: 234–252 pmol/8 × 10<sup>8</sup> RBCs). This exemplifies that the prediction of downstream response with an upstream marker will most likely yield a greater variability, which will eventually hamper the dosing decision. Recently, drug transporter protein *ABCC4* was found to be actively transporting 6-MP and 6-TGN from the hematopoietic cells, thereby protecting the cells in



**Figure 4** Different levels of variation observed during 6-MP treatment. As one moves from gene variant to clinical response, the downstream responses are dispersed for a given upstream genotypic/phenotypic variant. **(a)** For a few *TPMT* gene variants, several *TPMT* enzyme activities are observed on continuous scale in humans. **(b)** For a specific gene variant, there is a huge variation in *TPMT* activity and possible overlap with other gene variant. **(c)** Relationship between *TPMT* activity and 6-TGN concentration; for a given range of *TPMT* activity, a huge variation in 6-TGN concentration was observed. **(d–f)** Relationship between 6-TGN concentration and cellular response; for a given 6-TGN concentration, substantial dispersion in cellular responses were observed during 6-MP treatment. 6-MP, 6-mercaptopurine; 6-TGN, 6-thioguanine nucleotide; RBC, red blood cell; *TPMT*, thiopurine *S*-methyltransferase; WBC, white blood cell.



**Figure 5** 95% Confidence region for 6-TGN concentration predicted through nonparametric Bayesian population modeling approach. Black region: CR prediction based on genotypic information; gray: CR based on TPMT enzyme activity; red: CR based on 6-TGN measurement; solid dot: 6-TGN measurement. 6-TGN, 6-thioguanine nucleotide; CR, confidence region; RBCs, red blood cells; TPMT, thiopurine S-methyltransferase.

TPMT-deficient patients.<sup>51</sup> These findings demonstrate that although one can measure TPMT activity (molecular phenotype) precisely, there is a significant uncertainty in the level of 6-TGN (drug phenotype), the compound that is responsible for treatment response. In addition, when 6-TGN acts on the cellular population, another level of uncertainty is added which leads to different clinical outcome for similar 6-TGN concentration range. Hence, phenotyping enzyme activity or even 6-TGN concentration may not be sufficient; rather, the cellular response, which is the ultimate response variable of interest should be regarded as the basis for dose individualization. **Figure 4** summarizes these variations observed in various clinical studies. An extensive list of different covariates and their effect on variability on molecular and clinical phenotypes are included in the **Supplementary Materials**.

### FUTURE DIRECTIONS AND EMERGING TECHNOLOGIES

From the discussions in the previous sections, it is clear that DNA sequencing and gene expression profiles provide some vital information on pathophysiological and clinical response for a given treatment regimen; however, augmenting this information with downstream biomolecular and cellular responses will facilitate unequivocal, quantitative clinical decisions. The application of qualitative upstream information in decision making may lead to uninformed conclusions for a specific individual. What is needed instead is an integrative approach that takes into account different levels of potential variation in the drug–disease cycle to predict the clinical outcome of interest and is adaptive in nature to each individual patient. As such, it should be an ongoing process rather than a “study-and-adopt” approach. In other words, after the initial detailed study and accumulation of information, a basic

set of information must be obtained from each new patient in order to adapt the approach before making predictions on clinical outcomes. Given the dynamic nature of physiological responses, this naturally warrants the application of dynamic modeling and *in silico* approaches at a suitably sophisticated level. It is not only proactive in predicting the clinical response but also alleviates the need for continuous/frequent monitoring, which will be prohibitive from physiological, logistical, and economical points of view.

In recent times, there has been increasing recognition of the utility and practice of quantitative tools in medical applications.<sup>52,53</sup> At the same time, the tremendous increase in life science research over the last several decades has resulted in a system, which publishes thousands of relevant articles every year. Clearly unaided individual clinicians and healthcare practitioners are unable to process all these articles and incorporate into practice those advances that will have the greatest clinical impact. As such, there is a tremendous opportunity to develop a systematic approach to embedding scientific advances in clinical decision support tools. Although these tools have been largely statistical, the time is now opportune to expand this quantitative approach to include mathematical models and systems theoretic tools that embed scientific advances toward maximizing clinical impact. Mathematical models, suitably empowered by systems theoretic methodology, derive their strength from their potential to quantitatively evaluate known or conjectured mechanisms of medical cure. Although engineering and mathematical personnel can provide skillful use of quantitative tools, success of such endeavors is contingent on utilizing the judgment of experienced medical personnel. For such a closely integrated effort, collaboration must occur among medical and engineering researchers over an extended period in a clinical setting. A recent report by the National Academy of Engineering and the Institute of Medicine elaborates on how a partnership between healthcare professionals and engineers could change the face of the 21st century healthcare system.<sup>54</sup> A detailed road map was also laid to harness the power of systems engineering tools, information technology, and complement knowledge across scientific disciplines to achieve what was termed the “six Institute of Medicine quality aims” of the healthcare system that included safety, effectiveness, patient centeredness, timeliness, efficiency, and equitability.

Although gene expression information by itself is not ideal, systems biologists have developed methodologies to predict phenotypic outcomes for a specific gene expression pattern through the simulation of metabolic networks. These metabolic networks aid in linking pharmacogenomic variants, such as SNPs, to pathophysiological (phenotypic) outcomes. Through *in silico* models of these metabolic networks, the effects of sequence variations, alterations in specific components, and resulting biochemical reaction kinetics can be analyzed in the context of the rest of the reactions in the entire network.<sup>55</sup> Application of such an approach has been demonstrated for human RBCs using a large-scale metabolic network, in which *in silico* models predicted pathophysiological outcomes for two established SNPs associated with two key enzymes. As expected, no clear relationship was observed between the SNPs and their associated kinetic parameters. However, when evaluated in the context of other



simultaneously altered enzyme kinetics within the whole network, the model predicted overall cell behavior and eventually the clinical outcome.<sup>55</sup> Augmenting this approach with other omics data will uniquely identify the flux modes and further enhance the predictive power. We have developed a class of dynamic metabolic models, widely known as “Cybernetic Models,” which provides a framework to accommodate gene expression information and enzyme regulation and predict the system-level, dynamic metabolic profile and overall cellular outcome.<sup>56,57</sup> The potential clinical utilities of these metabolic models are evident from their ability to identify functionally interrelated sets of reactions and metabolites that are causally related to diverse pathophysiological conditions including xenobiotic metabolism and biomarker identification.<sup>58–60</sup> The power of such approaches lies in the automatic integration of patient-specific information, both genotypic and phenotypic, which leads to the prediction of entire metabolic flux profiles and overall cellular outcomes. These metabolic and cellular functions can readily be associated with observed efficacy and/or toxicity through statistical and mathematical tools, which will help in clinical decision making.

Recent advances in genome-scale computational models are also expected to provide key insights into how complex phenotypes evolve as a function of gene variants and molecular interactions. Genome-scale metabolic models are large-scale extensions to traditional metabolic networks to analyze the entire cell in the light of available data and computational methods. Mathematical representation of physiochemical, environmental, and regulatory constraints and computational solutions enable the identification of feasible and infeasible metabolic behavior, leading to reliable predictions even when comprehensive data is not available.<sup>61</sup> Using a computational model that accounted for all annotated gene functions, Karr *et al.*<sup>62</sup> have provided an understanding of several biological processes that were not feasible earlier through experimental techniques. In addition, the model has accurately predicted molecular pathologies of single gene disruption phenotypes. The reconstruction of tissue-specific, genome-scale metabolic models such as the ones describing human liver metabolism, cancer cell metabolism, along with an increased availability of extensive patient-specific “omics” data to refine these models, bodes well for advancing these approaches for personalized treatment.<sup>60,63</sup>

Often, it is not feasible to obtain objective, quantitative cellular response frequently as in the case of neuropathic pain, cancer progression etc., or the drug is so toxic to some patients that we cannot afford to titrate the drug dose. In these cases, measuring covariates that are closely connected (on drug–disease cycle) to the clinical response may provide acceptable surrogate information for clinical decision making. For example, small molecules qualify as the immediate effector of the clinical response.<sup>64</sup> Recently, a new concept of personalized treatment based on metabolomics phenotype has been proposed by Nicholson *et al.*<sup>65,66</sup> and termed as pharmacometabonomics. Metabonomics, a special case of metabolomics, studies the systematic variation in the metabolic profiles due to external stimuli such as genetic modification, biological stimulus, and xenobiotic intervention. Pharmacometabonomics, at the intersection of pharmacology and metabonomics, was defined as “the prediction of

the outcome, efficacy, or toxicity of a drug or xenobiotic intervention in an individual based on a mathematical model of a preintervention metabolite signature.” Pharmacometabonomics aims to study the global metabolic fingerprint in predose biofluids and characteristic change in the metabolic profiles due to drug dosing in the postdose biofluids. These two vital pieces of information can then be correlated to the clinical responses using chemometric tools. The key metabolites identified from this exercise are then mapped onto the relevant metabolic networks through various databases to reveal functional relationships in disease pathways. This approach, if designed carefully, promises to provide an unbiased and hypothesis-free analysis of the metabolic profile, which may help to identify unexpected biomarker combinations.<sup>65</sup> These endogenous metabolites will eventually aid in identifying patient subgroups that may be cured and/or are susceptible to side effects before commencing the treatment. A recent review article provides an extensive discussion of several preclinical and clinical applications of this new emerging area.<sup>67</sup>

Recent advancements in single-cell measurement and manipulation technologies allow multiscale and multiparametric measurement of molecular contents, thereby enabling observation of molecular events at single-cell level.<sup>68</sup> Elucidation of system-wide interaction of molecular and signaling events and comparison with the response of pathological cells under the influence of a therapeutic drug provide new quantitative mechanistic insights.<sup>69</sup> When combined with mathematical models, this information provides further insight into underlying mechanisms and important parameters that are unable to be extracted from experimental techniques. Extension of these single-cell models, accounting for deterministic and stochastic population heterogeneity, aid in prediction of overall physiological outcomes and emergence of multimodal cellular populations with distinct phenotypic features.<sup>44,70</sup> Apart from these detailed, global mechanistic approaches, several classes of semimechanistic models have been developed for various *in vivo* processes over the last few decades.<sup>53,71</sup> These types of models are adequate to describe a macrolevel phenomenon within the exhaustive overall process and predict cellular outcome for treatment intervention. However, rigorous efforts for individualization of these models and effective clinical translation are lacking.

Integrated quantitative approaches that combine genotypic, molecular profiling, and clinical data have shown promise in predicting causal relationships between specific genotypic/molecular signatures and biological and/or clinical outcomes.<sup>72</sup> Unlike traditional statistical approaches, which are often restricted to correlational studies, these approaches develop causative models, purely driven by data and prior knowledge, that establishes the dependence structure among various interacting biomolecular entities and have the ability to provide causal influence. Interactomics modeling has also shown promise to evaluate quantitative interactions at macromolecular level (DNA, RNA, proteins, and other molecules) and aid in understanding how local and global molecular network dynamics affect overall cellular properties and ultimately lead to human diseases.<sup>73</sup> Although some of these approaches have largely been in use for drug target discovery, or applied for other simpler

organisms, it is now time to gain insights from them and extend to personalized treatment based on an individual patient's omics signature.

The quantitative approaches discussed here are admittedly in their infancy and require extensive validation in carefully designed, prospective clinical studies. The key conundrum in this regard would involve translating these fast burgeoning scientific findings emerging from various fronts. Admittedly, it is difficult to verify each one of these findings in controlled clinical trials. An interesting option would be to integrate the detailed, mechanistic network models with PBPK-IVIVE (PBPK: physiologically based PK; IVIVE: *in vitro*, *in vivo* extrapolation) approach in which ADME (absorption, distribution, metabolism, and elimination) is managed by physiologically based pharmacokinetics-*in vitro*, *in vivo* extrapolation, whereas kinetic insights for physiologically based pharmacokinetics are governed by genetic and other omics data through metabolic network models.<sup>74,75</sup> Eventually, this approach could serve as a screener to select candidates for controlled clinical trials and design them. In addition, several other constraints surface while translating such approaches in routine clinical practice. One should consider logistical, computational, and economic factors. Efforts must also be diverted to effective communication of these techniques to clinicians and ensure ease of use and interpretation. Social, ethical, and privacy-related risks of such personalized approaches are not insignificant. Genetic minorities must be protected from discrimination by drug developers, insurance companies, and employers. Unfortunately, besides these quantitative approaches, there are not many alternatives available to solve current challenges in treating critical diseases. However, we believe that a concerted and collaborative effort by various stakeholders and experts will pave the way for effective implementation of holistic personalized medicine in clinical settings.

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