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## Opportunities and Challenges of Proteomics in Pediatric Patients: Circulating Biomarkers After Hematopoietic Stem Cell Transplantation As a Successful Example

Sophie Paczesny, MD, PhD<sup>1,\*</sup>, Christine Duncan, MD<sup>2</sup>, David Jacobsohn, MD<sup>3</sup>, Robert Krance, MD<sup>4</sup>, Kathryn Leung, M.D<sup>4</sup>, Paul Carpenter, MD<sup>5</sup>, Catherine Bollard, MD, PhD<sup>3,6</sup>, Jamie Renbarger, MD<sup>1</sup>, and Kenneth Cooke, MD<sup>7</sup>

<sup>1</sup>Indiana University School of Medicine, Indianapolis, IN, United States of America

<sup>2</sup>Boston Children's Hospital, Boston, MA, United States of America

<sup>3</sup>Children's National Medical Center, Washington DC, United States of America

<sup>4</sup>Texas Children's Hospital, Houston, TX, United States of America

<sup>5</sup>Fred Hutchinson Cancer Research Center, Seattle, WA, United States of America

<sup>6</sup>George Washington University, Washington, DC, United States of America

<sup>7</sup>Johns Hopkins University School of Medicine, Baltimore, MD, United States of America

### Abstract

Biomarkers have the potential to improve diagnosis and prognosis, facilitate targeted treatment, and reduce health care costs. Thus, there is great hope that biomarkers will be integrated in all clinical decisions in the near future. A decade ago, the biomarker field was launched with great enthusiasm because mass spectrometry revealed that blood contains a rich library of candidate biomarkers. However, biomarker research has not yet delivered on its promise due to several limitations: (i) improper sample handling and tracking as well as limited sample availability in the pediatric population, (ii) omission of appropriate controls in original study designs, (iii) lability and low abundance of interesting biomarkers in blood, and (iv) the inability to mechanistically tie biomarker presence to disease biology. These limitations as well as successful strategies to overcome them are discussed in this review. Several advances in biomarker discovery and validation have been made in hematopoietic stem cell transplantation, the current most effective tumor immunotherapy, and these could serve as examples for other conditions. This review provides fresh optimism that biomarkers clinically relevant in pediatrics are closer to being realized based on: (i) a uniform protocol for low-volume blood collection and preservation, (ii) inclusion of well-controlled independent cohorts, (iii) novel technologies and instrumentation with low analytical sensitivity, and (iv) integrated animal models for exploring potential biomarkers and targeted therapies.

\*Correspondence: Sophie Paczesny, MD, PhD, Indiana University, 1044 W. Walnut Street, Room R4-425, Indianapolis, IN, 46202, Phone: 317-278-5487, Fax: 317-274-0138, sophpacz@iu.edu.

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## 1 Introduction

The identification and validation of biomarkers can contribute to major advances in the development of new therapies. The main types of biomarkers are diagnostic, predictive, and prognostic. They can be used to more accurately diagnose a disease, personalize treatment, identify novel targets for drug discovery, and enhance the efficiency of drug development. Biomarkers are identified through a wide range of approaches that include genetics, proteomics, immunomics, and metabolism. This review presents a viewpoint on biomarker development, discusses relevant analytical considerations, and provides a regulatory perspective summarizing a pathway toward biomarker validation.

Although written to encompass all aspects of biomarker discovery, validation, and qualification, this review centers on biomarkers of graft-versus-host disease (GVHD) due to recent advances in related biomarker development. Allogeneic hematopoietic stem cell transplantation (HSCT) is an increasingly widely used therapy in a range of malignant and non-malignant hematologic diseases. In allogeneic HSCT, the recipient immune and bone marrow systems are replaced by the donor immune and hematopoietic stem cells, with both positive and negative consequences. In malignant disease, the donor immune system can recognize residual leukemic cells as foreign and eradicate them by immunological means called the graft-versus-leukemia (GVL) effect. However, donor immune cells may also attack normal host tissue, particularly the skin, liver, and gastro-intestinal tract, resulting in the GVHD reaction [1]. The occurrence of GVHD remains one of the major barriers to more widespread and successful application of HSCT. Furthermore, a major barrier to GVHD research and treatment is that the diagnosis and prognosis rely almost entirely on the presence of clinical symptoms, which are sometimes confirmed by biopsy. Currently, no laboratory tests exist to predict the risk of developing GVHD, responsiveness to treatment, or patient survival. Despite these obstacles, considerable efforts have been made to develop GVHD biomarkers in a way that approaches used for GVHD biomarker discovery can now be considered as examples to follow. Indeed, the ability to identify patients at high risk for GVHD early in their transplantation and treatment course has important therapeutic implications, indicating when more stringent monitoring and/or preventative care will be beneficial. The ability to identify patients who will not respond to standard treatment and who are at particularly high risk for subsequent morbidity and mortality could result in personalized treatment plans, such as additional immunosuppressive treatments, that might be more effective if introduced early for high-risk patients. The identification of patients who will respond well to treatment could allow for more rapid tapering of steroid regimens, thereby reducing long-term toxicity and allowing a more robust GVL response in low-risk patients.

The current review provides an update on the different types of biomarkers in the age of omics, the types of samples to be collected with a focus on the pediatric population, omics approaches for the discovery of circulating biomarkers, as well as a summary of successful approaches used to obtain biomarkers for acute GVHD.

## 2 Types of biomarkers in the age of omics

The NIH Biomarkers Definition Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, NIH [2]). The Institute of Medicine further defined “objectively” to mean “reliably and accurately” [3]. Biomarkers are ideal for “high-risk” translational studies involving mechanistically heterogeneous diseases [4] (add smoking cessation ref), with GVHD occurrence being a prime example.

Biomarkers have been evaluated in both hypothesis-driven and discovery-based testing for specific clinical outcomes. Initially, most biomarkers were identified from mechanistic studies and correlated with a particular outcome. Most recently, several groups have begun exploring omics biosignatures and biomarkers. Biomarkers can be classified into five types: (1) diagnostic, (2) predictive, (3) prognostic, (4) pharmacodynamic, which is a dynamic assessment showing that a biological response has occurred after a therapeutic intervention, and (5) surrogate disease, which is a substitute for a clinical endpoint. Due to the absence, so far, of studies investigating pharmacodynamic and surrogate markers correlated to GVHD, this review focuses specifically on the first three types of biomarkers, which may have the most significant short-term translational impact (**Box 1**).

## 3 Blood and plasma sample collection for molecular profiling

Success in molecular profiling is highly dependent on sample quality and standardized operating procedures. Some profiling technologies, such as those involving microRNAs, require relatively large sample volumes that may be a limiting factor in the pediatric population. Noninvasive clinical tests are to be preferred because it allows for repetitive sample collection from the same patient over a short period of time. Therefore, biofluids, such as plasma, sera, urine, or proximal fluids from an affected organ such as bronchoalveolar lavage (BAL) for lung are samples of choice. Effort has been made to develop standardized methods for clinical sample collection [5, 6]. The Human Proteome Organization (HUPO) Plasma Proteome Project specimen collection and handling report found that: (1) platelet-depleted plasma is preferable to serum for certain proteomic studies; (2) the addition of protease inhibitors should be incorporated early and used judiciously, as some form nonspecific protein adducts and others interfere with peptide studies (most plasma repositories to date do not add protease inhibitors); (3) diligent tracking is needed for pre-analytical variables, such as venipuncture vs. phlebotomy vs. from an existing line, tuning using a gel or non-gel separator, for plasma the nature of anticoagulant (EDTA, heparin, or citrate), processing time and protocols, storage conditions, and number of freeze/thaw cycles; and (4) the use of reference materials for quality control and quality assurance also should be considered. These findings help provide guidance on sample handling issues,

with the overall recommendation that researchers must be conscious of all possible pre-analytical variables as a prerequisite of any proteomic study.

Since most biorepositories have available plasma and sera, these are the biofluids most frequently analyzed. Unfortunately, plasma and sera contain many different proteins with a wide dynamic range, spanning twelve orders of magnitude from the most abundant to the lowest abundance protein. Clinically interesting proteins, such as cytokines and their receptors, are usually present in the lowest abundance [7, 8]. Detection of these low abundance proteins requires depletion of the predominant proteins and extensive fractionation of the proteome [9].

Urine and proximal biofluid samples [BAL, cerebrospinal fluid (CSF), saliva, etc.] represent alternatives to plasma and sera samples for biomarker identification. These samples are far less complex and can be obtained in large quantities. But, the limitations associated with their use are that they yield better information about diseases in the organs directly involved in their production and excretion, such as the kidneys, lung, brain, mucosa, etc., and often the samples are diluted and a method for normalizing the samples is required.

Peripheral blood mononuclear cells (PBMCs) have also been used by immunologists to discover cellular biomarkers and more recently immunomics as discussed below. Unfortunately, repositories do not always contain as many PBMCs as compared to plasma or sera.

Investigating markers in the damaged tissue itself could be more informative than screening systemic markers and therefore collection and banking of biopsies of GVHD target organs such as skin, intestine, liver, lung, and mucosa should be encouraged as often as possible. As mentioned above, good alternatives are biofluids of GVHD target organs such as bronchial lavage fluid (BAL) for the lung, saliva for the oral mucosa in the context of chronic GVHD, as recently published [10, 11].

Proteomic studies will be designed based on the availability of biofluids in biorepositories. Many individual transplant centers have established biorepositories to collect and store serial samples from patients at their centers. These are very useful for biomarker discovery efforts and initial validation of specific biomarkers. However, there has also been an effort to establish larger biorepositories associated with national multicenter trials. These biorepositories offer the great advantage that samples are collected from multiple centers and validations using these samples are therefore less likely to reflect center-dependent variables. Validations using these large multi-center repositories will be more likely to gain clinical acceptance and impact clinical practice. Of note, a limitation specific to the pediatric population is the limited volume available for serial blood collections. Thus, one future solution would be the use of dried blood/plasma spot cards, which allow for smaller sample volumes, are easier to handle, and are less expensive to transport and store. This approach is currently being explored [12], and we expect the use of such cards to be implemented in the next 5 years.

## 4 Omics approaches for discovery of circulating biomarkers

Several omics approaches can be utilized for discovery of circulating biomarkers and are summarized below and in Figure 1 and **Box 2**.

### 1. Proteomic biomarkers

In this review, we focus specifically on the use of proteomics for the diagnosis of GVHD following HSCT, because proteins are more proximal to the ongoing pathophysiology of a disease [13]. The proteome varies with time and is defined as “the proteins present in one sample at a certain point in time.” Both non-mass spectrometry (MS)- and MS-based proteomic approaches have been employed to discover biomarkers. Antibody-based approaches rely on the unique properties of antibodies that can bind to an extremely wide range of molecules with exceptional binding specificity that enables the measurement of picomolar amounts of proteins in blood samples. On the other hand, MS-based approaches have become a powerful tool for characterizing and assessing both qualitative and quantitative changes in complex protein mixtures [14]. Several MS techniques are used in clinical proteomics: matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS, liquid chromatography (LC) [9, 15–17], and capillary electrophoresis (CE) [18, 19]. These are gel-free methods that have several advantages over gel separation such as better separation of proteins with a low molecular weight and, shorter analysis time. Fractionation is followed by MS. This analytical procedure reliably identifies proteins and determines their isoforms and post-translational modifications. MS also allows quantification, especially when tandem MS (MS/MS) is used [20][9, 15, 21]. In addition, new instrumentation allows for better accuracy of the peptides identified [22]. The next step is the matching of mass spectra to a sequence database to identify peptides and proteins [23].

Although secreted proteins have been a fundamental source for biomarker discovery, it is important to note that protein biomarkers in serum and plasma often exist in multiple states and they may include multimers with different functional activity as well as states bound to other proteins. These different states are often not known or measured but may profoundly affect the clinical relevance of the measured biomarker. Recent efforts have been made to measure the functional states of proteins by identifying and understanding Protein post-translational modifications (PTMs). PTMs increase the functional diversity of the proteome by the covalent addition of functional groups or proteins, proteolytic cleavage of regulatory subunits or degradation of entire proteins. These modifications include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis and influence almost all aspects of normal cell biology and pathogenesis. In addition, these PTMs result in number of protein variants (isoforms) showing micro heterogeneity of different kinds and high impact on the biological/ pathological function [24]. Proteomics approaches are also used for validation of candidate biomarkers. Immunoassays remain the assays of choice for validation, but high-quality antibodies pairs for sandwich enzyme-linked immunosorbent assay (ELISA) with the required affinities and specificities for the target are rare, leading to bias in the prioritization of candidate markers. ELISA assays are simple and highly reproducible between users and between laboratories, limiting both inter- and intra-assay variability. The two main disadvantages of validation by

ELISA are: 1) the large volume of patient plasma required, and 2) the bias introduced by selection of candidate biomarkers with commercially available ELISA kits. To overcome the first problem, multiplexing technology is preferred in the absence of cross-reactivity. To overcome the second problem, selected reaction monitoring-MS (SRM-MS) also called multiple reaction monitoring (MRM) is a potentially solution [25–28]. SRM-MS is used for targeted, multiplexed quantitative proteomics. Currently immunoassays are the standard for biomarker validation, but SRM may quickly a strong alternative, especially if recommendations from the NIH workshop addressing targeted peptide measurements in biology and medicine are implemented [29]. Furthermore, constrained SRM is ideal to quantify PTMs and isoforms [30].

## 2. Immunologic biomarkers

Immunomics is the study of immune system regulation in response to physiological or pathogenic processes. Unprecedented sophistication in instrumentation and software tools for human immunology studies have allowed researchers to visualize high-dimensional data in biological networks and evaluate the interrelationships between genes and/or proteins [31]. Recently, mass cytometry has addressed the analytical challenges of polychromatic flow cytometry by using metal atoms as tags rather than fluorophores as well as atomic MS as the detector instead of photon optics. The stable isotopes can provide up to 100 distinct tags that can be measured simultaneously in a single experiment. Using mass cytometry, the Nolan's lab (Stanford) [32] were the first to show that perturbation of responses in individual cancer cells could be related to clinical outcomes in leukemia patients. An advantage of this single-cell approach is that signaling can be characterized in rare populations of cancer cells and compared with the bulk cancer cell population. The study from Stanford revealed and characterized the cell signaling heterogeneity of acute myeloid leukemia (AML) and showed that signaling in individual cancer cells can be closely linked to the clinical behavior of the disease [32]. Moreover, mass cytometry was applied to analyze differential immune responses to different drugs [33].

However, one of the most important obstacles facing research of the human immune system is the much greater inter-individual and intra-individual diversity compared with that of inbred mice. Experiments in animals infected with a particular virus and housed in pathogen-free facilities allow for detection of T cells expressing a particular T-cell receptor known to be specific for a particular virus epitope presented by a major histocompatibility molecule known to be expressed in that mouse strain. If the same experiment is now performed in humans, a whole new dimension of genetic and environmental complexity is added. Indeed, humans are outbred and thus express unknown combinations of human leukocyte antigen (HLA) molecules, and their environment is rich in pathogens. Therefore, individuals contain unknown varieties of T cells specific for unknown varieties of viral peptide-HLA complexes, requiring high-dimensional computational and technologic power. However, recent advances in single-cell evaluations, flow cytometry and high-throughput TCR sequencing mean that we now have improved access to the unique tools required to comprehensively analyze the adaptive T-cell immune responses in pediatric patients after HSCT [34].

### 3. Metabolic biomarkers

The metabolome is defined as the full complement of small-molecule metabolites found in a specific cell, organ, or organism. Metabolomics is expected to have an impact in the areas of cancer, stem cell research, and the development of drug resistance. Relative to other omics measurements, metabolomics provides a more immediate and dynamic picture of the functionality of a cell. Furthermore, an important advantage of metabolomics is that unlike genomics and proteomics, humans and mice largely share the same metabolites. Therefore, identification of metabolic biomarkers in mouse and human samples relies on the same search engines. However, its use is currently limited to specialized laboratories because of the difficulty to measure the metabolome due to a fast reaction (seconds) to the environment, the large dynamic range of metabolites, the absence of amplification process, and the lack of fluorescent labeling without altering their normal function. MS is the analytical methodology of choice for identifying metabolites. It is likely that improvements leading to more complete coverage of the metabolome will emerge soon and that metabolomics will be part of our large collection of biomarkers in the near future, [35].

### 4. Nucleic acid-based biomarkers

Since the advent of next-generation sequencing (NGS) particularly DNA sequencing in 2005, there has been several studies employing the technology to tackle previously intractable questions in many disparate biological fields. This has been coupled with technology development occurring at a remarkable pace. Hematologic malignancies are at the forefront of cancers whose genomes have been the subject of NGS. The first cancer genome to be sequenced by NGS was from a patient with a cytogenetically normal AML [36]. Multiple driver mutations have since been identified in AML and other hematologic malignancies such as myelodysplastic syndrome, myeloma, chronic lymphocytic leukemia, and solid cancers. These studies have opened novel areas of biology that can be exploited for prognostic, diagnostic, and therapeutic means.

Another important application of NGS is the study of the diversity of receptors that govern antigen recognition. Hence, we can now study normal and pathologic T and B cell repertoires, as well as monitor rare T-cell populations such as  $\gamma\delta$  T cells. This will allow, for example, an unprecedented depth of analysis of immune reconstitution following HSCT, which is particularly important in the pediatric population. Altogether, this increasingly affordable technology will undoubtedly impact future practice and care of patients with hematologic malignancies and following HSCT [37].

DNA analyses are not limited to findings of mutated DNA. Other strategies include quantification of methylated DNA or microRNAs (miRNAs). Hypermethylation of the CpG islands in the promoter region of tumor suppressor genes has been considered a contributing event in carcinogenesis via the silencing of suppressor gene transcription. Many of these targets are also found to be hypermethylated in the plasma of cancer patients and can serve as circulating biomarkers [38]. miRNAs are small (20–25 nucleotides) noncoding segments of RNA, such as that function by binding to mRNAs and regulating gene expression. Because they are released into the circulation and they are relatively resistant to degradation, miRNAs may be useful blood-based biomarkers [39, 40]. However, one limitation is that

current methodologies require a large volume of plasma to detect miRNAs. They have been evaluated in GVHD [41].

### **5. Circulating cells or DNA as tumor biomarkers**

As noted above, circulating immune cells can be extensively phenotyped. Two technologies are currently used: (i) conventional flow cytometry measuring fluorescent labels [42, 43], and (ii) mass cytometry measuring metal probes with minimal signal overlap common to atomic MS [44].

Recently circulating tumor cells (CTCs) were identified and used as cancer biomarkers [45]. A number of techniques to capture CTCs have been developed using epithelial cell adhesion molecule (EpCAM) antibodies. Their role as early-stage cancer biomarkers is currently being explored [46, 47].

As noted in the previous paragraph, next generation DNA sequencing can also be used to detect and quantify tumor cells. This biomarker is being applied to quantify levels of minimal residual disease after stem cell transplantation as well as after other therapies. In this setting the target of sequencing can be rearranged B or T cell receptors in lymphoid malignancies or specific clonal mutations in other cancers [37].

### **6. Experimental models to discover biomarkers and as a tool for drug development**

The paucity of biomarkers resulting from human-based discovery studies is due in part to the limited availability of appropriate quality biospecimens that overcome potential collection variability. The acquisition of human samples that are most pertinent to the intended clinical application represents another challenge. Particularly, samples that are the most relevant for early detection applications are difficult to obtain. However, blood and tissue collected at early stages of disease development would be readily available using experimental models including genetically engineered mouse models. This will allow discovery of biomarkers in relation to disease characteristics and over time. In addition, mice are maintained under conditions that minimize environmental biases [48, 49].

Animal models may also be used for drug development (Figure 2). Animal models can be utilized early, in the “target validation” stage of drug development, when studies are needed to understand the target and determine if it meets the criteria for a drug screen. Second, when pharmacologic agents are found, they can be tested in animal models to evaluate their potential efficiency and toxicity.

### **5 Computational analysis for high-dimensional data**

Current datasets include many variables as well as multiscale data [different locations, types of molecules (proteins, miRNA, or cells, etc.), and time points]. Bayesian probability offers a statistical approach that captures the “causal” relationship between variables. Causal networks are constructed using conditional correlations based on “prior” information. “Priors” include data such as known protein-protein interactions, and transcription factor binding sites. Bayesian methods infer causal relationships between molecular relationships by randomly generating many possible network models and using statistical techniques to

select a consensus model that best fits the data [50]. Thus, these methods balance the trade-off between prior knowledge and the data. Many of the software programs used to integrate high-throughput datasets are based on Bayesian principles [31]. These mathematical models based on networks interactions have also been referred to as ‘systems biology’. Systems biology build comprehensive, multiscale network models that accurately capture all of the regulatory elements of a biological system (e.g., immune system). Maps of potential connections among the variables are generated and may suggest new functional roles for specific genes, proteins, or metabolites. For biologists already focused on a particular molecule of interest, These analyses may potentially place a molecule of interest in the context of new pathways, molecular interactions, tissues, or diseases, allowing for new hypotheses to be tested. Predicted connections are then modified to test and validate the model through in vitro assays and in vivo experiments. This approach has also been used with datasets for small molecule drugs and allows for the discovery of repurposed drugs [51].

## 6 Statistical considerations

### 1. Framework for biomarker evaluation

Guidelines for reporting on diagnostic accuracy studies and prognostic studies have been proposed. The standards for Reporting of Diagnostic Accuracy (STARD) were published in 2003 [52], and REporting recommendations for tumor MARKer prognostic studies (REMARK) were published in 2005 [53]. Recently, the Institute of Medicine (IOM) proposed a framework for biomarker evaluation that comprises three components [3]:

- 1) Analytical validity. This step requires analyses of the analytical performance of an assay. Performance characteristics include (i) precision (repeatability, reproducibility); (ii) accuracy; (iii) assay sensitivity (i.e., limit of detection); (iv) assay specificity (i.e., interference and cross-reactivity); (v) sample type and matrix; (vi) sample preparation and conditions; (vii) performance around the cutoff; and (viii) potential for carryover and cross-hybridization (mostly for multiplex technology).
- 2) Qualification.. This step requires assessment for associations between biomarkers and disease states, including data showing the effects of interventions on both the biomarker and clinical outcome.
- 3) Utilization. This step requires a contextual analysis based on the specific use proposed and the applicability of available evidence for this use. This includes a determination of whether the analytical validity and qualification conducted provide sufficient support for the use proposed.

The US Food and Drug Administration (FDA) has established a framework for biomarker development and qualification in the Center for Drug Evaluation and Research that is more stringent than the one proposed by the IOM [54, 55] (Figure 3). In the FDA framework, the biomarker is qualified for a specific “context of use”. A context of use is defined as a “comprehensive and clear statement that describes the manner of use, interpretation, and the purpose of a biomarker in drug development”.

## 2. Statistical considerations

Several statistical methods can be used, and examples are summarized in **Box 3** and detailed below. The target condition (condition of interest) refers to a well-defined clinical disorder such as a particular disease, a disease stage, or a condition that triggers a clinical action such as the beginning, modification, or termination of a treatment.

### a. Diagnostic accuracy statistics

- **Sensitivity.** The sensitivity of a new test is estimated as the proportion of subjects with the target condition in whom the test is positive. For example, a sensitivity of 85% for a GVHD diagnosis test will correctly predict the presence of GVHD in 85% of patients tested.
- **Specificity.** The specificity of a new test is estimated as the proportion of subjects without the target condition in whom the test is negative. For example, a specificity of 85% for a GVHD diagnosis test will correctly predict the absence of GVHD in 85% of patients tested.
- **Receiver operating characteristic (ROC) analysis.** The ROC curve is a plot of the true-positive rate (sensitivity = 1 – false negative error rate) versus the false-positive rate (1 – specificity) for all possible thresholds [56, 57]. Whereas sensitivity and specificity can only be calculated with a defined threshold, the ROC curve can be used to calculate true and false positive rates for all possible thresholds[58].
- **Positive predictive value (PPV).** The proportion of patients with a positive test who have the target condition.
- **Negative predictive value (NPV).** The proportion of patients with a negative test who do not have the target condition.

### b. Biased estimate

Abiased estimate is one that is systematically too high or low. Bias creates inaccurate estimates [59], and often the existence, size (magnitude), and direction of the bias cannot be determined. However, understanding potential sources of bias may help avoid or minimize bias.

- c. **Selecting the study population.** Bias can be seen when a study does not include the complete spectrum of patient characteristics or when patient subgroups are missing. Thus, the “real world” performance of the test for the intended use has to be evaluated. If the study population is all inclusive, the test is likely to meet the validity criteria. Recommendations to satisfy external validity are to use the final version of the test, have multiple users with relevant training and expertise run several tests, and use a range of operating conditions.
- d. **Sample size.** Statistical power and number of specimens that should be tested have to be determined before the study starts. Traditional sample size calculations have been challenged by Pepe et al. who proposed that computer simulations based on

biological models and number of cases and controls should determine the best sample size for the study in question [60].

## 7 Graft-versus-host disease biomarkers

All different types of biomarkers are represented in GVHD, as it is not only a systemic immunological disorder but it also affects specific organ systems such as the skin, gastrointestinal tract, and liver during the acute phase, and mucosa during the chronic phase. Several teams have discovered and validated circulating biomarkers relevant to HSCT in both hypothesis-driven and discovery-based testing. The data have come primarily from single centers or from a number of collaborating centers; in most cases, the findings have not been assessed as part of large multicenter trials. In Table 1, we summarized published GVHD biomarkers that were examined in more than one study and/or as a drug target and/or more than one cohort. Of this list, nine biomarkers (CXCL9, Elafin, HGF, IL-2Ra, IL-8, Keratin 18, Reg3a, ST2, TNFR1) [16, 17, 61–65] have been validated in an independent cohort using the same assay. Some are currently commercially available to clinicians with the limitation that thresholds for different clinical outcomes have still to be established in prospective collected samples. Details on the discovery and validation of these biomarkers were recently reviewed [66–69]. The next steps include evaluation of these biomarkers with samples collected in a multicenter cohort, which is necessary for reduction of center effects and the successful design of subsequent trials. This direction has already been taken by the Blood and Marrow Transplant Clinical Trial network that is currently acquiring prospective samples to validate thresholds and intend to pursue with therapeutic intervention for newly diagnose GVHD patients. This successful biomarkers identification, validation and implementation approach develop for GVHD could serve as an example for advances in other pediatric fields.

## 8 Conclusions

In a short-time, omics technologies have made tremendous progresses and have allowed identification of novel candidate biomarkers or molecular pathways. In the field of HSCT, the use of proteomics has led to the identification of novel biological mechanisms, which were unlikely to have been discovered by traditional hypothesis-driven research. Unlike gene expression levels, protein levels may be influenced by several post-transcriptional modifications and other factors, such as the cytokine milieu, requiring validation and qualification of biomarkers in different settings. Once a biomarker-based risk stratification algorithm is established, personalized medicine will be possible. In addition, the biomarker findings presented above offer the potential for exploring targeted therapeutics. Figure 4 summarizes the workflow of the development of biomarkers from the bedside to the bench and back to the bedside.

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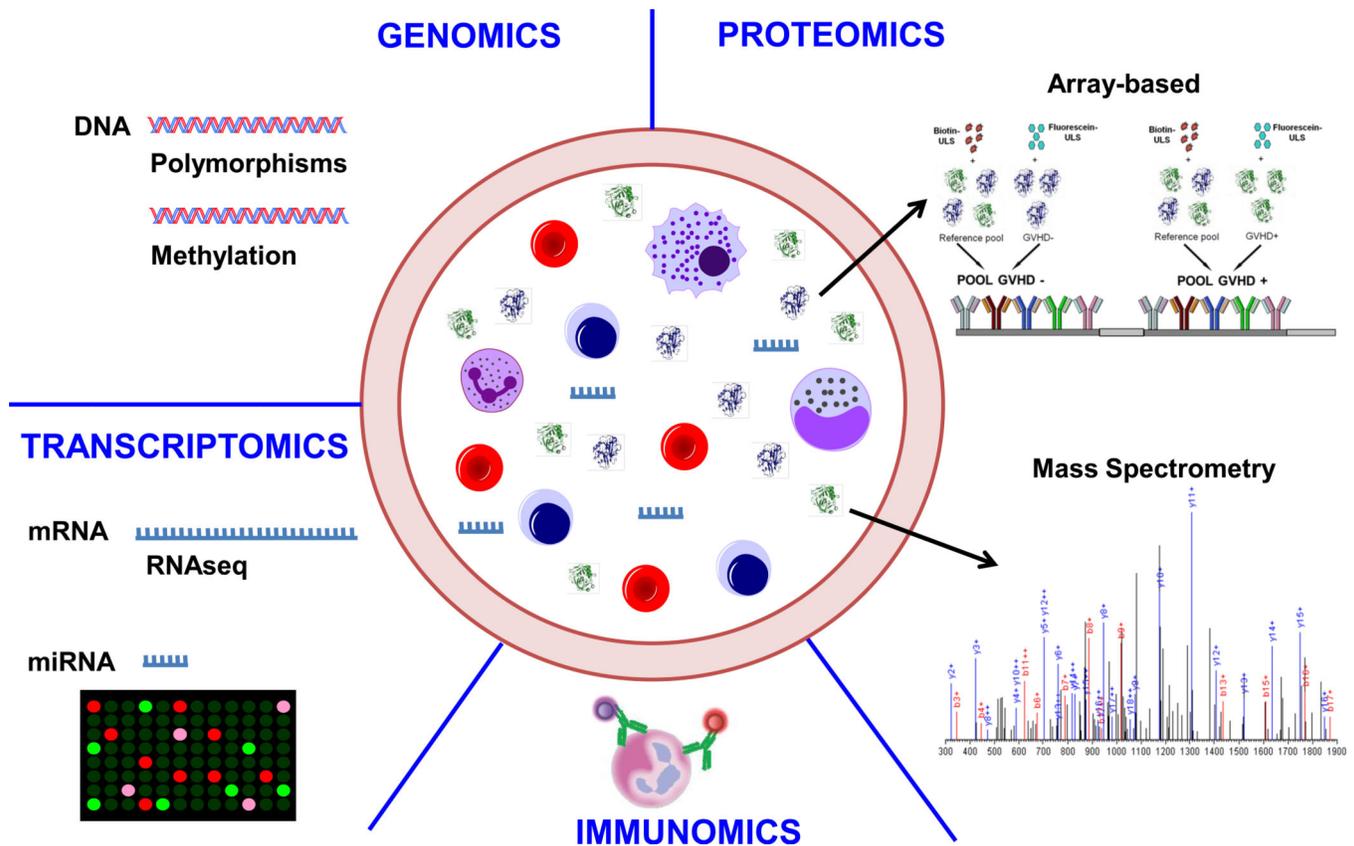
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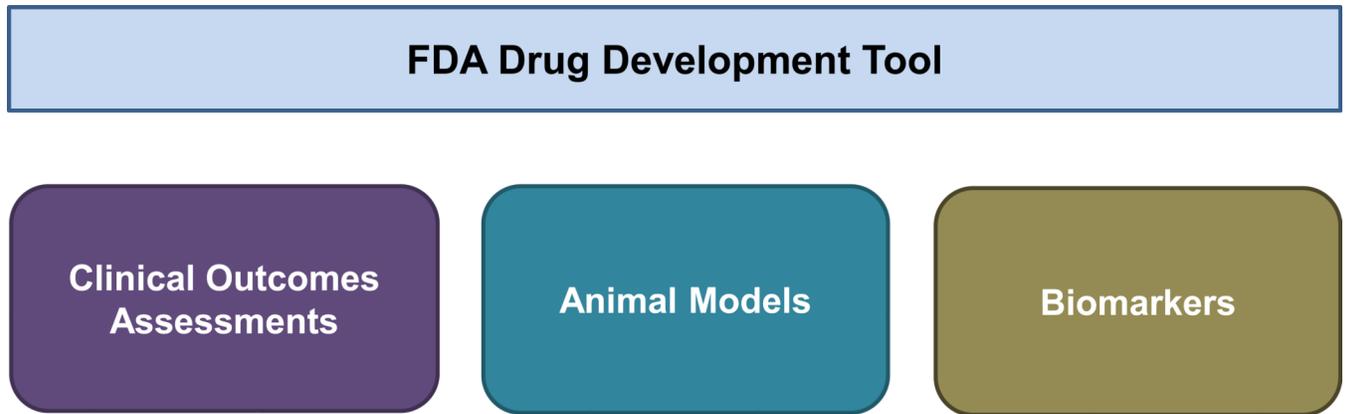
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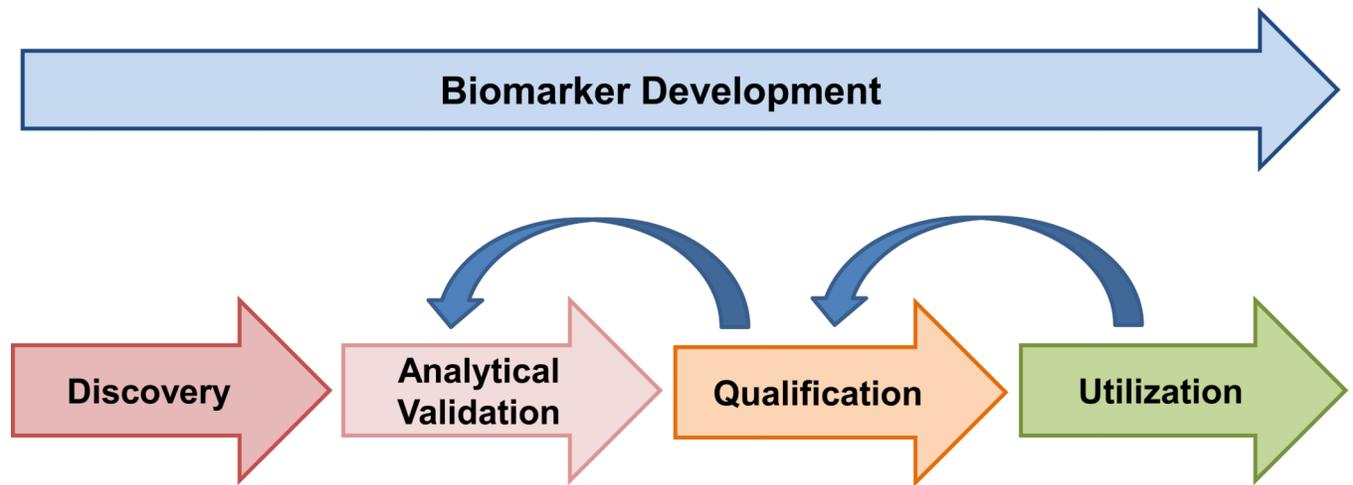


**Figure 1. Assessment of circulating biomarkers through blood omics**

A large number of high-throughput molecular and cellular profiling omics tools can be used to profile circulating biomarkers. Genomic approaches consist of determining gene sequence and function (for example, genome-wide association studies, RNA interference screens, exome sequencing, and methylation). Transcriptomic approaches consist of measuring the abundance of cellular or plasmatic RNA and also microRNAs present in cells or serum/plasma. Cytomics is used to phenotype immune cells based on intracellular or extracellular markers using polychromatic flow cytometry or mass cytometry. Proteomics approaches are based on either multiplex antibody assays or mass spectrometry.

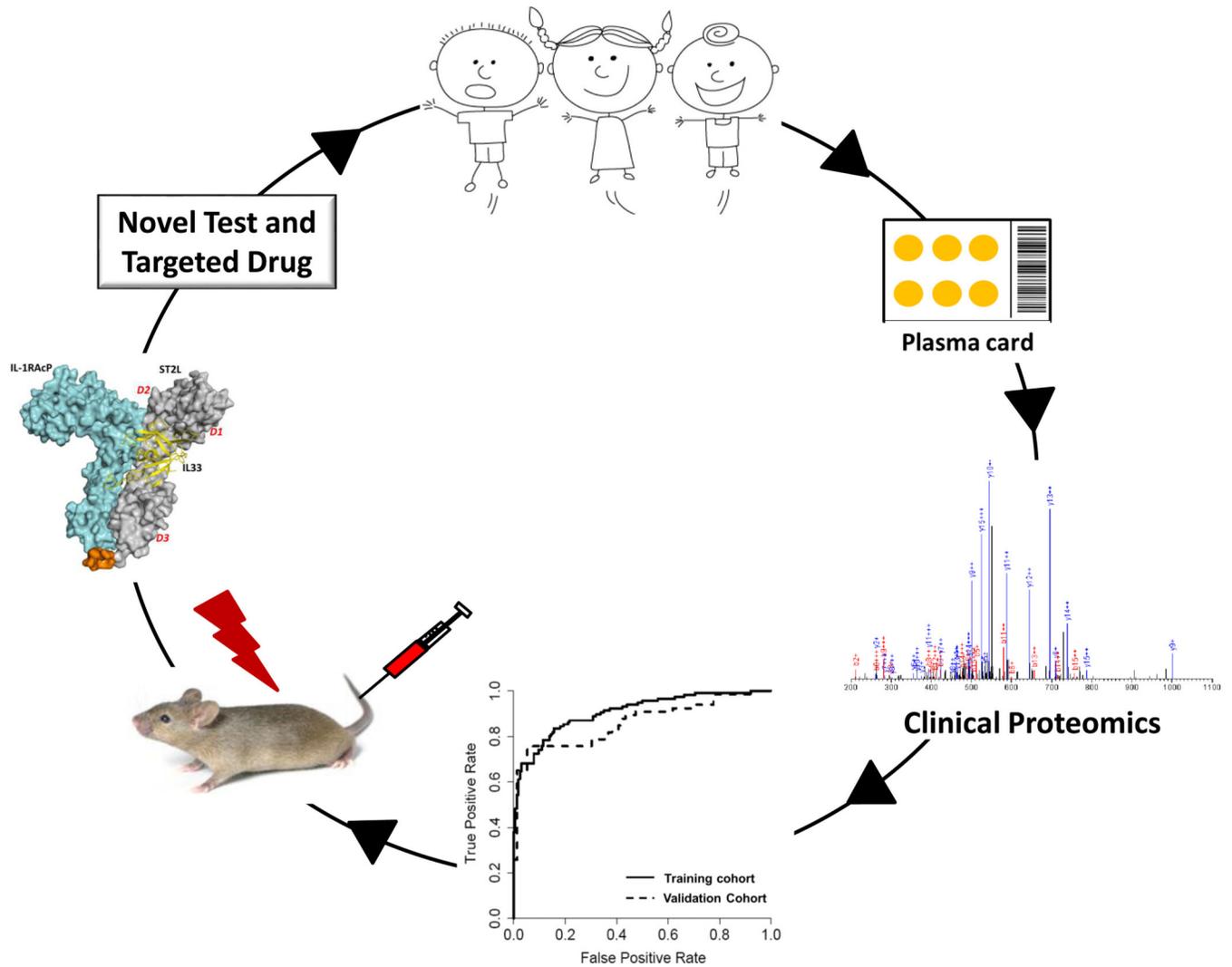


**Figure 2. US Food and Drug Administration (FDA) drug development tools (DDTs)**  
The DDT qualification Program, which includes biomarkers, was created by the Center of Drug Evaluation and Research to guide and prepare drug development efforts for rigorous safety and efficacy testing and eventual regulatory evaluation.



**Figure 3. Data from biomarker discovery (e.g., “omics” projects) can be used to focus further efforts along each step of validation to utilization**

Biomarker development is interdependent. From discovery, a candidate biomarker is initially measured and analytically validated for its precision and accuracy. An optimal measure would be practical (e.g., robust and cost-effective) and easy to collect (e.g., noninvasive, measured with surrogate tissue such as saliva). A validated test is required before qualification, which is the linkage of the measure with its clinical outcome. Finally, independently corroborated qualification of biomarkers can be “utilized” for more general purposes.



**Figure 4. Workflow for the discovery, qualification, and implementation of new biomarkers** Samples (blood or plasma) are obtained from children diagnosed in the clinic with GVHD, ideally as dried spots in the near future. The proteins in the sample are subjected to separation and purification followed by MS/MS for protein identification. The protein concentrations from the patients' samples are then compared to known concentrations of the identified protein in an immunoassay (usually sandwich ELISA). Once a biomarker is validated in a training and validation cohort and shows good sensitivity and specificity, its ability as a diagnostic, predictive, and/or prognostic tool is evaluated in clinical trials. Biomarkers may also offer important insights into the biology of a disease and identify novel pathways. Moreover, biomarkers may offer the potential for use in exploring targeted therapeutics. The end goal is more personalized treatment and improved patient outcomes.

Table 1

## Candidate GVHD biomarkers

Biomarker	Studies	No. of patients in studies	Role in GVHD
<b>Proteomic biomarkers</b>			
$\alpha 4\beta 7$ Integrin	Chen 2009 [70]	59	Increased expression on naïve and memory T cells prior to intestinal aGVHD.
Calprotectin	Rodriguez-Otero 2012 [71] Chiusolo 2012 [72]	72 68	Increased levels at the time of GI-GVHD diagnosis are predictive of treatment responsiveness.
C reactive protein (CRP)	Schots 2002 [73] Pihusch 2006 [74] Malone 2007 [75]	96 350 147	CRP levels reflect the risk of transplant-related mortality and major transplant-related complications. Some studies show no difference between aGVHD and other complications.
CXCL9*	Croudace 2012 [76] Kitko 2014 [61]	46 355	Plasma CXCL9 elevations correlate with chronic GVHD diagnosis and a decrease in circulating CD4(+) cells expressing CXCR3 in the blood, whereas increased in tissue biopsies of the dermis.
Elafin <sup>a)</sup>	Paczesny 2010 [17] Levine 2012 [77]	522 112	Produced by keratinocytes. Increased concentrations at onset of skin aGVHD.
Hepatocyte growth factor (HGF) <sup>a)</sup>	Okamoto 2001 [78] Paczesny 2009 [62] Harris 2012 [63] Levine 2012 [77]	38 424 954 112	Increased in patients with aGVHD.
Interleukin (IL)-2R $\alpha$ <sup>a)</sup>	Miyamoto 1996 [79] Foley 1998 [80] Seidel 2003 [81] Visentainer 2003 [82] Shaiegan 2006 [83] Paczesny 2009 [62] August 2011 [84] Levine 2012 [77]	30 36 60 13 67 424 62 112	Early post-transplantation levels are high prior to clinical signs of aGVHD.
IL-6	Mohty 2005 [85] Pihusch 2006 [74] Malone 2007 [75]	113 350 147	Elevated at onset of aGVHD.
IL-7	Dean 2008 [86] Thiant 2010 [87] Thiant 2011 [88]	31 40 45	Levels increase significantly post-transplantation with an inverse correlation to absolute T-cell count.
IL-8 <sup>a)</sup>	Ugucioni 1993 [89] Schots 2003 [90] Mohty 2005 [85] Paczesny 2009 [62] Levine 2012 [77]	30 84 113 424 112	Early increase post-transplantation is associated with increased mortality. Some studies show no difference between aGVHD and other complications.
IL-18	Fujimori 2002 [91] Shaiegan 2006 [83]	14 67	Correlates with the levels of IL-2R $\alpha$ .
Keratin 18 <sup>a)</sup>	Luft 2007 [64] Luft 2011 [92] Harris 2012 [63]	55 48 954	Increased levels during hepatic and intestinal aGVHD that correlate with treatment unresponsiveness.
Regenerating islet-derived protein 3 $\alpha$ <sup>a)</sup>	Ferrara 2011 [16] Levine 2012 [77]	1034 112	Released into circulation via GVHD-induced breaches of the mucosal intestinal barrier. Elevated in GI-GVHD.
Suppression of tumorigenicity 2 (IL-33 R $\epsilon$ ) <sup>a)</sup>	Vander Lugt 2013 [65]	1054	Increased levels at initiation of therapy correlate with treatment unresponsiveness and mortality. Elevation early post-transplantation (day 14) is associated with increased risk for death.

Biomarker	Studies	No. of patients in studies	Role in GVHD
Tumor necrosis factor (TNF)- $\alpha$ / TNF-receptor 1 <sup>a)</sup>	Holler 1990 [93]	56	Elevation early post-transplantation is associated with severe aGVHD and treatment-related mortality.
	Abdallah 1997 [94]	80	
	Schots 2003 [90]	84	
	Mohty 2005 [85]	113	
	Choi 2008 [95]	438	
	Paczesny 2009 [62]	424	
	August 2011 [84]	62	
Levine 2012 [77]	112		
Peptide Sets	Kaiser 2004 [18]	45	2013 study: aGvHD urinary proteomic classifier of 17 spectra
	Weissing 2007 [96]	175	
	Weissing 2013 [19]	423	
<b>Cellular biomarkers</b>			
CD30	Hubel 2010 [97]	30	Increased plasma levels and expression on T cells are associated with the severity of aGVHD.
	Chen 2012 [98]	53	
Dendritic cells (DCs)	Lau 2007 [99]	40	Decreased total blood DC count is associated with severity of aGVHD.
	Akhtari 2010 [100]	25	
Invariant natural killer T (iNKT) cells	Chaidos 2012 [101]	57	Low iNKT/T cell ratio is associated with the occurrence of aGVHD.
	Rubio 2012 [102]	71	
Regulatory T cells	Rezvani 2006 [103]	32	Levels are inversely correlated with the severity of aGVHD.
	Wolf 2007 [104]	58	
	Magenau 2010 [105]	215	
	Koreth 2011 [106]	23	
	Di Ianni 2011 [107]	28	
	Matsuoka 2013 [108]	45	

Examined in more than one cohort and/or more than one study and/or as a drug target

<sup>a)</sup> Biomarkers using the same assay validated in an independent cohort