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Clinical proteomics for post-hematopoeitic stem cell transplantation outcomes

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Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is the most effective form of tumor immunotherapy available to date. However, while HSCT can induce beneficial graft-versus-leukemia (GVL) effect, the adverse effect of graft-versus-host disease (GVHD), which is closely linked to GVL, is the major source of morbidity and mortality following HSCT. Until recently, available diagnostic and staging tools frequently fail to identify those at higher risk of disease progression or death. Furthermore, there are shortcomings in the prediction of the need for therapeutic interventions or the response rates to different forms of therapy. The past decade has been characterized by an explosive evolution of proteomics technologies, largely due to important advances in high-throughput mass spectrometry instruments and bioinformatics. Building on these opportunities, blood biomarkers have been identified and validated both as promising diagnostic tools, prognostic tools that risk-stratify patients before future occurrence of GVHD and as predictive tools for responsiveness to GVHD therapy and non-relapse mortality. These biomarkers might facilitate timely and selective therapeutic intervention. This review summarizes current information on clinical proteomics for GVHD as well as other complications following HSCT. Finally, it proposes future directions for the translation of clinical proteomics to discovery of new potential therapeutic targets to the development of drugs.

I- Introduction

Allogeneic hematopoietic cell transplantation (HSCT) is the most effective form of tumor immunotherapy available to date. However, while HSCT can induce beneficial graft-versus-tumor/leukemia (GVT/L) effects, the adverse effect of graft-versus-host disease (GVHD), is the major source of morbidity and mortality following HSCT. Until recently, available diagnostic and staging tools frequently failed to identify those at higher risk of disease progression or death. Furthermore, there are shortcomings in the prognosis and stratification of patients at risk before the clinical signs, and in the prediction of the response rates to different forms of therapy. The past decade has been characterized by an explosive evolution

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Competing interests. S.P. is an inventor on a patent entitled “Methods of detection of graft-versus-host disease” (US 20130115232A1, WO 2013066369A3). Jochen Metzger is employee of the biotechnology company Mosaiques Diagnostics GmbH located in Hannover/Germany, which developed the CE-MS technology.

of '-omics' technologies, largely due to important advances in chemistry, engineering, high-throughput instrumentation and bioinformatics. Building on these opportunities, blood biomarkers have been identified and validated both as promising diagnostic tests, as prognostic tests that stratify patients at risk of future occurrence of GVHD, and as predictive tests for responsiveness to GVHD therapy and non-relapse mortality. These biomarkers might facilitate timely and selective therapeutic intervention. However, such blood tests are not yet available for routine clinical care. Below we will summarize current information on clinical proteomics that have been used to identify and validate biomarkers of GVHD and other post-transplantation complications.

II- Definitions and types of clinical biomarkers

A biomarker, typically a protein, is defined as a characteristic that can be objectively measured and evaluated as an indicator of a normal biologic process, pathogenic process, or pharmacologic response to a therapeutic intervention [1].

The need for biomarkers in the context of post-hematopoietic stem cell transplantation (HSCT) is due to the limitations of current predictors. Known risk factors pre-HSCT are related to genetic factors, including HLA disparities between donor and recipient, age, unrelated transplant, conditioning regimen intensity, malignant disease status, and donor graft source and content. A diagnosis of acute graft-versus-host disease (aGVHD) post-HSCT relies on clinical signs in one of three major target organs, skin, liver, and/or gastrointestinal (GI) tract [2], and can be confirmed by biopsies of these organs in these fragile patients. In addition, histologic severity on biopsy has not been consistently correlated with clinical outcome [2]. Furthermore, dynamic risk factors after HSCT should be considered as possible contributing or confounding factors such as levels of immunosuppression, prophylactic and interventional use of antibiotics, occurrence of infections, and changes in the microbiome diversity as mentioned later.

In the past decade, various types of biomarkers have been identified. The 2014 NIH Chronic GVHD Consensus Biomarker Working Group, which included GVHD as well as U.S. Food and Drug Administration experts, defined the different types of biomarkers and summarized an ideal framework for biomarker development [3]. There are 4 types of markers: 1) Diagnostic biomarkers identify the presence of a disease as compared to similar presentation of other etiology (i.e. aGVHD of the gut versus infectious colitis), 2) Prognostic biomarkers categorize patients by degree of risk for disease occurrence before the clinical signs appear, 3) Predictive biomarkers are used to categorize patients by their likelihood of response or outcome to a particular treatment when measured before the treatment, and 4) Response to treatment markers differentiate between patient populations that have responded or not to a particular treatment (i.e., favorably or unfavorably), as opposed to patients who did not have that response. Predictive biomarkers after HSCT will also indicate maximum GVHD severity, nonrelapse mortality (NRM), and overall survival (OS). In the context of HSCT, two categories of markers have been prioritize for their potential to guide therapeutic decisions and ultimately survival: 1) prognostic biomarkers measured early in the course of transplantation that predict occurrence of GVHD prior to clinical signs allowing for preemptive treatment, and 2) predictive biomarkers measured before the treatment that will

allow for intensification of treatment in the high risk group and decreased immunosuppression in the low risk group.

II Biological fluids of interest and sample collection post-HSCT

Ideal clinical tests are based on noninvasive collection, which allows for repetitive collection of samples from the same patient in a short amount of time. GVHD biomarkers may be produced by several sources, such as donor cells, the local or systemic cytokine milieu, or recipient target tissues during disease development, or by interaction of the microbiome and its host. These proteins may then be released into a variety of body fluids. For noninvasive tests used for diagnostics or screening, biofluids, such as plasma, sera, or urine, or stools are the preferred samples. Collection of samples is the initial step of the analytical procedure and affects the chances of obtaining relevant data in the search for specific biomarkers. The various steps from patient sampling to sample storage should be considered potential sources of artifacts in any experimental design. Therefore, an enormous effort has been made to develop standardized methods of clinical sample collection for proteomic studies [4]. Biospecimen reporting for publications should include appropriate informed consent, conditions of biospecimen collection, and sample processing. The sample processing should include tube types; additives such as anticoagulants, preservatives, and protease inhibitors, if used; quality control standard operating procedures; information management with inventory control and tracking; storage and distribution conditions, such as storage temperature and length of storage; number of freeze/thaw cycles; and variations in collection and processing across biospecimen sets.

Blood is the most frequently analyzed bodily fluid, and the ease with which it can be sampled makes it a logical choice for biomarker applications. The levels of individual blood proteins represent a summation of multiple, disparate events that occur in every organ system. Blood contains proteins shed by the affected tissue as well as proteins that reflect secondary systemic changes. In addition, the blood proteome depends on many other factors governing the actual state of the whole organism that may not be related to the monitored disease, complicating the evaluation and pertinence of the data obtained. Another factor that complicates the analysis of plasma/sera is the wide range of protein amounts and isoforms. Plasma and sera are highly complex mixtures containing high amounts of many different proteins with a wide dynamic range, spanning 12 orders of magnitude from albumin to the lowest abundance, often most clinically relevant, proteins such as cytokines and their receptors [5]. The 22 most abundant proteins constitute approximately 99% of the plasma proteome, whereas the remaining 1% of the plasma proteins are medium and low abundance proteins [5b]. Thus, both depletion of the predominant proteins and subsequent fractionation of the proteome are usually required to allow the detection of low abundance proteins. Unfortunately, the steps involved in sample preparation may result in the loss of proteins of interest during the depletion step [6]. Considering that most clinically relevant plasma biomarkers belong to the low abundance plasma protein fraction and have concentrations 10 [5b, 6-7] times lower than those of albumin [5a], highly sensitive detection methods are required.

Urine samples represent an alternative to plasma/sera samples for biomarker discovery. Urine has three main advantages compared to plasma/sera: (i) it can be obtained in large quantities; (ii) the protein mixture is far less complex and the variation in protein abundance is low [7]; and (iii) it is more stable than plasma [8]. However, a limitation is that urinary proteins are produced mainly from kidney function (~70%) and only partially by glomerular filtration of plasma proteins (~30%) [7].

Stool samples post-HSCT have been valuable samples for biomarkers; their advantage is being in proximity to the mucosa of the main target organ.

An ideal schedule of sample collection post-HSCT will contain both calendar- and event-driven collection. Based on currently validated biomarkers, we propose a cost-effective collection for plasma/sera that contains calendar samples: pre-HSCT, day 7, day 14, day 21, day 28, and day 100, day 180, day 360 post-HSCT that should capture early events such as idiopathic pneumonia syndrome (IPS), sinusoidal obstruction syndrome (SOS, thrombotic microangiopathy (TMA), acute GVHD, and chronic GVHD (cGVHD). Event-driven samples should include onset of complications (i.e. SOS, aGVHD) during the 48-h window of treatment initiation, and onset of other complications that can either mimic GVHD or pose a difficult diagnosis. Sample quality, acquisition, and storage should be followed as specified above.

III Current clinical proteomics technologies

Antibody or Aptamers -based multiplex approaches have been widely used in different formats: microarrays or mass cytometry. The majority of nonantibody proteomics strategies are based on mass spectrometry (MS) which has become a powerful tool for characterizing both qualitative and quantitative changes in complex protein mixtures [9]. Gel-free separation methods, such as capillary electrophoresis (CE) and liquid chromatography (LC) are currently utilized.

1) Antibody-based microarrays

Antibodies have unique characteristics derived from their three important properties: (i) their ability to bind to an extremely wide range of natural and man-made chemicals, biomolecules, and cells; (ii) their exceptional binding specificity that enables the measurement of picomolar (10−12) amounts of proteins in blood samples; and (iii) the strength of binding between an antibody and its target [10]. However, we should note two major limitations to antibody-based assays: 1) in the context of arrays there is a limit to the number of antibody that can be spotted without cross-reactivity between the different antibodies, and 2) in the context of single assays, they could be a problem of comparability between different assays depending on the antibody used, thus the necessary step of validation of the test with a one assay and to lock down the test as mentioned in the framework of biomarker development [3]. To screen for aGVHD biomarkers, antibody microarrays dotted with hundreds of antibodies have been employed, allowing hundreds of proteins in complex biological matrices to be measured [11]. Using antibody microarrays comparing patients with and without aGVHD as a discovery engine and ELISA to validate the lead candidates, the description of a four-protein biomarker panel [interleukin (IL)-2

receptor α chain (sIL-2Rα/sCD25), tumor necrosis factor receptor-1 (TNFR1), IL-8, and hepatocyte growth factor (HGF)] for aGVHD diagnosis [12] opened the field of large scale biomarkers after HSCT. An example of antibody-based microarrays used for aGVHD diagnosis is shown in Figure 1.

2) Aptamer-based microarrays

Aptamers are short single-stranded oligonucleotides or peptides that are capable of binding various molecules with high affinity and specificity. The company SomaLogic developed the SOMAscan platform that could identify and quantify 1,129 analytes simultaneously. This was available to researchers through cores. The platform has not been used in the post-HSCT context but has been in systemic scleroderma, a condition closely related to sclerodermic cGVHD of the skin; ST2 and Spondin-1 were their top biomarkers validated in three independent cohorts [13].

3) Mass cytometry, or CyTOF

CyTOF is a time-of-flight (TOF) mass spectrometry that is used like flow cytometry but in which the antibodies are labeled with heavy metal ion tags instead of fluorochromes. The main advantage as compared to flow cytometry is that it allows for the combination of more antibody specificities in a single sample (classically 30–50 antibodies) without significant spillover between channels. This technology has been used in combination with computational tools, especially unsupervised algorithms. It organizes and displays high dimensional data in a way not possible with traditional, supervised gating techniques [14] providing a new opportunity to comprehensively characterize cellular heterogeneity and discover new populations. One such algorithm, viSNE, has been shown to be robust in its ability to distinguish both healthy and disease subsets, showing great promise for research and clinical analysis and visualization of cytometry data [14c, 15]. viSNE creates a phenotypic map of cells from an individual sample, or collection of samples, enabling visualization of phenotypic relationship between individual cells [14c, 16]. Given viSNE's particular strength in visualization of high dimensional single cell data, it is well-suited to identify subtle or large changes in marker expression across several samples. Additionally, cells with unexpected phenotypes are routinely overlooked in manual analysis and viSNE captures many of these overlooked cells [14c, 17]. Most advances with this technology occurred in studies of blood and bone marrow, since the cells in these tissues are readily obtained in single-cell suspensions. The Irish lab has identified several cGVHD patterns using CyTOF [18]. Recently a group compared flow cytometry and CyTOF in the context of cGVHD [19].

4) CE-MS technology (capillary electrophoresis coupled to mass spectrometry)

Capillary electrophoresis on-line coupled to mass spectrometry (CE–MS) has been applied by Weissinger and colleagues since 2003 to identify urinary peptide biomarkers for early detection of acute GVHD (aGVHD) [20]. In the following years, candidate biomarkers were combined to an aGVHD-specific support vector machine-based multivariate classifier, named aGVHD_MS17 that allows distinction of patients with severe aGVHD from those who never develop aGVHD and patients with low or moderate aGVHD. The characteristic CE-MS peptide marker profiles for the different patient groups are presented in Figure 2.

Recently the predictive value of aGVHD_MS17 was evaluated in two large prospective studies. The first study was conducted with the objective to detect severe aGVHD 21 days in advance to clinical signs. This study included 463 leukemia patients after stem cell transplantation between 2005 and 2010 enrolled in five German transplant centers. In this study, it was found that aGVHD_MS17 enabled the diagnosis of aGVHD grades III and IV with a sensitivity and specificity of 72.6 and 78.6 % when diagnosis was exclusively based on the proteomic pattern and 82.4 and 77.3 % when the proteomic pattern was combined with relevant demographic and clinical variables [21]. In 2008, a multicenter, randomized, placebo-controlled, double blind clinical trial was started to test the applicability of aGVHD_MS17 to the clinic as a tool to predict severe aGVHD and to initiate pre-emptive steroid therapy (Trial registration: ISRCTN03911524). Eleven German transplant-centers contributed 267 patients to this trial and 92 were randomized according to the positivity of aGVHD-MS17 to receive either the steroid prednisolone or placebo. As reported at the ASH meeting in 2017, prospective and blinded evaluation of aGVHD_MS17 in this trial revealed that the first analysis time point at 2–17 days after HSCT was most accurate in the prediction of aGVHD ≥ grade II with a sensitivity of 87% and a specificity of 81%. Sensitivity and specificity remained high until day 30 post-HSCT, but then the pattern lost its predictive value at later time points which could be attributed to late effects of reduced intensity conditioning and early death after stem cell transplantation.

A second proteomic peptide marker pattern was established by the same research group in 2008 for early diagnosis of cGVHD, to differentiate it from acute GVHD, and to predict its onset and severity. The cGVHD_MS14 classifier was prospectively evaluated over a time period of 9 years on 422 patients at four German transplant centers. Prospective and blinded evaluation revealed the correct classification of patients developing cGVHD with a sensitivity of 92.5% and a specificity of 75.3% [22]. Acute GVHD prior to day 100 is not recognized by cGVHD-MS14 and its classification factors are correlated to the severity of cGVHD according to the Seattle limited and extensive cGVHD nomenclature.

5) LC-MS/MS technology

Two fundamental strategies for protein identification and characterization by MS/MS are currently employed in clinical proteomics. In bottom-up approaches, complex protein mixtures, are subjected to proteolytic cleavage, and the peptide products are analyzed by MS. The proteins can first be immunodepleted of the most abundant plasma proteins (i.e., albumin, IgG, IgA, transferrin, haptoglobin, and anti-trypsin), and also separated by chromatography, in which case the sample will contain only one or a few proteins. In top-down proteomics, intact protein ions or large protein fragments are subjected to gas-phase fragmentation for MS analysis. Furthermore, new instrumentation, such as the ultra-high-resolution linear ion trap Orbitrap mass spectrometers, facilitates top-down LC–MS/MS and versatile peptide fragmentation modes [23], increasing the number of proteins identified. The mass spectra are then matched to a sequence database to identify proteins [24]. Both top-down and bottom-up approaches have been used to identify GVHD markers. The Intact Protein Analysis System (IPAS) is a top-down method. It was used to compare GVHD-negative and -positive pools of 10 patients matched for other clinical characteristics. Intact proteins were then labeled on cysteine residues with heavy and light acrylamide-stable

isotopes. The two pools were combined, and specimens were subjected to multiple fractionations. The individual fractions were then digested and analyzed on a LC–MS/MS instrument [25]. Due to the labor intensity and cost of the IPAS workflow, the current trend is to use bottom-up approach with iTRAQ labeling (isobaric tags for relative and absolute quantification) [26] or Tandem Mass Tag (TMT) labeling even though it detects less low abundance proteins. Figure 3 summarizes top-down and bottom-up approaches.

IV Validation of biomarkers and Major phases of biomarker development

The SOMAscan platform, CE-MS technology, and CyTOF have been used at the discovery and validation steps. Multiple-reaction monitoring has emerged as a potentially useful technique for validation [27]. This rapid tandem mass spectrometric technique enables the targeted monitoring and quantification of candidate molecules in complex samples. However, sandwich enzyme-linked immunosorbent assay (ELISA) remains the preferred method for validation because it is highly specific and employs two antibodies specific for the candidate protein, high-throughput, and cost-efficient as compared to the clinical proteomics validation tools. The procedure is also relatively simple and highly reproducible from performer to performer and from laboratory to laboratory, limiting both inter- and intra-assay variability. Efforts are underway to advance the omics technologies and to bring them to a technological level where they can compete with the ELISA technology.

The development of biomarkers entails a number of phases, from the identification of promising molecular targets to longitudinal clinical trials in association with a specific treatment. Five major steps are required to develop a clinical test for use in standard practice. First, the pilot phase compares 20 to 40 cases and controls using technologies as discussed above. It is recommended that the term “candidate biomarker” or “potential biomarker” be used to refer to findings of early phase studies when additional validation is needed. At the discovery-phase level, recommendations for biomarker identification and qualification in clinical proteomics have been proposed to avoid overinterpretation of results, the use of inappropriate technologies or statistics, and inefficiency in the construction of a multimarker panel [3, 28]. For reporting on observational study design and diagnostic accuracy, two guidelines have been established by the STROBE and STARD initiatives [29]. The number of specimens that should be tested depends on the objective of the study and the extent of biomarker variability in the study. As suggested by Pepe et al. [30], there are no simple methods for recommending sample sizes. In particular, traditional sample-size calculations that are based on statistical tests of hypotheses are not relevant, and they propose that computer simulations guide the choice of sample sizes, meaning that simulations should be performed with the guidance of investigators on biologically plausible models to generate data. By varying the numbers of cases and controls, one can assess at what sample size a reasonable proportion of promising biomarkers is likely to be selected for further study. Several statistical methods can be used to estimate the diagnostic likelihood ratio of a continuous biomarker, but Receiver operating characteristics (ROC) curves are primarily used [31]. ROC curves estimate the probability of disease given data on candidate marker(s) which is maximized at every cut-off [32]. Over the years, proteins with AUC of ROC > 0.70 have been considered for the next validation step. Second, there is the validation phase where the samples are taken from retrospective longitudinal case–control repository. This

process should ideally be done on a training set followed by a validation set if there is only one cohort, as was the case in our first biomarker panel [12]. At this stage, the analytical validity of the tests (accuracy) and practicality (non-invasive, cost) are also evaluated. The third step focuses on a few candidates for which assays have been considered to be analytically valid and use of samples from a large independent verification cohort from all comers (vs. selected patients). The fourth step requires to test the assay for one focused outcome on a prospective multicenter cohort, typically on thousands of samples, before the release of the assay. This step defines the cut-offs for the biomarker(s). It is hoped that this step will lead to a clinical test that will be approved by the U.S. Food and Drug Administration. Fifth, the impact of the test on the reduction of disease burden on the population of interest is quantified through biomarker-based clinical trials [30]. If all the steps above are passed, the test(s) could be used in standard practice. Importantly, the clinical context of use for a specific biomarker and specific outcome as to be defined up-front during the discovery phase [3]. Indeed, aGVHD and to a greater extend cGVHD are not homogeneous diseases, and time post-HSCT, organ manifestations, conditioning (myeloablative versus dose reduced), immunosuppression, etc… may matter. Therefore, the discovery cohort determines the biomarker to be validated. Each biomarker for each specific outcome has to follow the workflow of biomarker development all the way through including verification in prospective clinical cohort. This framework of biomarker development is shown in Figure 4.

V Recent validated post-transplantation markers

Biomarkers of Acute GVHD

Some biomarkers of aGVHD have already been described above while mentioning the technologies used to identify and validate them. Further, there are recent reviews that have described the most useful biomarkers [33]. Therefore, we will just summarize the most promising focusing on biomarkers identified through clinical proteomics and those that had also been validated in one or more independent cohorts. Several plasma biomarkers that correlate with clinical outcomes after allogeneic HSCT have been identified: a four-protein biomarker panel (IL-2Rα, TNFR1, IL-8, and HGF) for aGVHD diagnosis [12]; the soluble form of Stimulation-2 (ST2), the interleukin (IL)-33 receptor with therapy-resistant acute GVHD and non-relapse mortality (NRM) [25c, 34]; regenerating islet-derived 3-alpha (Reg3α) and T cell immunoglobulin mucin-3 (TIM3) with gastrointestinal acute GVHD [34b, 35]; elafin with skin GVHD [25b, 36]; HGF and cytokeratin-18 fragments (KRT18) with liver GVHD [35c, 37]. Clearly ST2 has emerged as the most validated plasma biomarker for aGVHD either measured alone [25c, 34a, 38] or in combination with other markers [34b-d]. Furthermore, ST2 has now been tested and validated on several platforms such as nonmyeloablative conditioning [39], in cord blood transplantation [34a], in HLA-haploidentical or HLA-matched transplantation with use of cyclophosphamide post-transplant [38]. ST2 has also been validated as a prognostic marker for aGVHD and NRM in large cohorts when measure as early as day 7 [40] or day 14 [25c, 34b] post-HSCT. Besides these plasma biomarkers and as described in detail above urinary peptide marker patterns have also been identified and validated as biomarkers for acute and chronic GVHD, respectively [20–22].

Intestinal tract bacterial floral diversity, as represented by the inverse Simpson index, was suggested as a risk-stratification biomarker. Fecal specimens were collected from 80 allo-HSCT recipients at stem cell engraftment, and the low-diversity group (inverse Simpson <2) had the highest rate of transplant-related death [41]. A similar approach was used to stratify patients at risk for relapse after HSCT [42]. The presence of specific species such as *Blautia* that correlate with reduced death from GVHD has also been proposed as a potential biomarker.[43] Microbiome-host interactions and their potential as biomarkers were recently and extensively reviewed by Andermann et al [44]. Low urinary levels of indoxyl-sulfate, a metabolite of indole that reflects GI microbiome diversity, has been correlated with poor outcome in a single-center cohort of 131 patients [45].

Biomarkers of other early complications post-HSCT

Hepatic sinusoidal obstruction syndrome (SOS), previously known as veno-occlusive disease, is one of the major complications during the early period after HSCT. The disease is caused by both toxic injury of conditioning therapy to sinusoidal endothelial cells and inflammation, initiating the clinical symptoms of hyperbilirubinemia, tender hepatomegaly, ascites, and weight gain. The incidence and severity of SOS have decreased significantly in recent years, but fatal outcomes of SOS are still observed in clinical practice. Biomarkers for the diagnosis of SOS [ST2, angiopoietin-2 (ANG2), L-Ficolin, hyaluronic acid (HA), and vascular cell adhesion molecule-1 (VCAM1)] and prognosis of SOS [L-Ficolin, HA, and VCAM1] have been identified through a proteomics study and validated in several cohorts [34c, 46]. ST2 has also been shown to be a prognostic biomarker of endothelial injury that link aGVHD and thrombotic microangiopathy (TMA) [47]. A recent study showed that ST2 and IL-6 are diagnostic and prognostic biomarkers of idiopathic pneumonia syndrome (IPS) compared to unaffected controls, whereas TNFR1 served as a diagnostic marker when compared to viral pneumonia. ST2 had the highest positive predictive value (PPV) with 50% PPV at onset and 25% at day 7 post-HSCT for IPS occurrence [48]. New-onset post-transplantation diabetes mellitus (PTDM) occurs commonly after HSCT and is associated with inferior survival. A recent study showed that high ST2 at engraftment predicted increased PTDM and NRM risk, independent of conditioning and grades 3–4 aGVHD [49].

Chronic GVHD biomarkers

Chronic GVHD (cGVHD) is the most common long-term complication of allo-HSCT and is a leading cause of mortality in patients that survive 2 years without relapse [50], limiting the wider application of this therapeutic approach to patients with hematologic malignancies and bone marrow failures. In contrast to aGVHD, cGVHD often presents with clinical manifestations that resemble those of autoimmune diseases, such as scleroderma, Sjogren's syndrome, and systemic lupus erythematosus [51]. The median onset of cGVHD is between 4 and 5 months post-HSCT. There are many risk factors for the development of cGVHD, including age at transplantation, donor source and HLA disparity, peripheral blood grafts, and a history of prior aGVHD [52]. Depending on the presence or absence of these risk factors, the rates of cGVHD can be as high as 40–70% [53]. Despite the high incidence of this complication, the pathophysiology of this disorder remains poorly understood. Its diagnosis is based on clinical symptoms (i.e., inflammatory and fibrotic components) of several target organs (e.g., skin, nails, mouth, eyes, genitalia, musculoskeletal, GI tract, liver,

and lung) that can be confirmed by biopsies. At present, no simple diagnostic or prognostic test for cGVHD exists. However in 2014, the National Institutes of Health cGVHD Biomarker Consensus Group and the Biology group summarized the state of the art for cGVHD biology and biomarkers [3, 54]. Blood biomarkers have been evaluated through hypothesis driven study such as high levels of soluble B-cell activating factor (sBAFF) [55] and the balance of B-cell subsets during B-cell reconstitution [reviewed in [56]] or the prolonged imbalance of $CD4^+CD25^+FOXP3^+$ regulatory T cells versus conventional $CD4^+$ T cells following HSCT has been associated with a loss of tolerance and significant cGVHD manifestations [57]. Only few noteworthy biomarkers publications that are listed below used a discovery proteomics approach in the pilot phase. Using a quantitative bottom-up proteomics approach, a biomarker panel of four proteins [ST2, CXCL9, matrix metalloproteinase 3 (MMP-3), and osteopontin] had significant correlation with cGVHD diagnosis. Moreover, when measured at day +100 after HSCT it allowed patient stratification according to risk of cGVHD [26c]. MMP-3 also correlated to BOS diagnosis [26b]. In a recent study, both CXCL9 and CXCL10 were significantly correlated in multivariate analysis with cGVHD diagnosis in the first replication cohort, but only CXCL10 in the second [58]. In another recent study gene expression profiling of circulating monocytes from cGVHD patients found significant up-regulation of IFN-inducible (including CXCL10) and damage-response genes in cGVHD patients as compared to controls. These pathways were further confirmed in plasma ELISA assays showing elevated levels of CXCL9 and CXCL10 [59]. Altogether, the IFN-inducible chemokines CXCL9 and CXCL10, responsible for CXCR3 expressing Th1/NK lymphocyte recruitment into tissues, are upregulated at diagnosis [59–60] and are worth to being pursued and tested in prospectively collected samples. Plasma CD163 concentration has been associated with de novo-onset cGVHD [61]. Using a combination of flow and mass cytometry, it was shown that patients with more severe cGVHD had lower mucosal-associated T cell frequencies, with a concomitant higher level of CD38 expression on T cells [19]. Last but not least, a multivariate marker pattern composed of 14 naturally occurring urinary peptides (upon which are fragments from thymosin β−4, eukaryotic translation initiation factor 4γ2, fibrinogen β-chain or various collagen chains) allowed early and accurate prediction of cGvHD before the onset of clinical signs in a prospective and longitudinal survey using 1253 urine samples of 412 post-HSCT patients [22].

VI From proteomics to discovery of first-in-class small molecules inhibitors

Biomarkers represent promising targets for new therapeutics. In addition, we propose that the discovery of GVHD-specific drugs based on biomarkers will target the appropriate effector T cells to both increase efficacy and lower toxicity. This approach represents the first step in a continuum of research that is expected to lead to the development of pharmacologic strategies to specifically treat GVHD. Recent therapeutic advances to treat aGVHD include a clinical trial combining an IL6 receptor targeting antibody (tocilizumab, approved to treat rheumatoid arthritis) with standard prophylaxis [62] and the breakthrough therapy designation for aGVHD of a JAK1/2 inhibitor (ruxolitinib, developed to treat myelofibrosis (MF) [63]) by Food and Drug Administration in 2016 [64]. For ruxolitinib, an

overall response rate of 45%, lower than the published 85% [64] and adverse effects requiring platelet transfusions were recently reported in children with steroid refractory aGVHD [65] hinting at the need to redefine its dose-limiting toxicity in different patient populations. Ruxolitinib resistance and failure have also been observed in MF patients [66]. Though promising, these new agents were developed for different diseases and subsequently adapted to target nonspecific effector cells inducing aGVHD by suppressing T cell activation and proliferation. Administration of an ST2 neutralizing antibody in the mouse aGVHD models, effectively reduces plasma sST2 levels, improves survival in mice and preserves GVT activity in HSCT [67]. The maintained or increased accessible IL-33 pool led to induce type 2 cytokines secretion as well as more regulatory T cells to ameliorate aGVHD progression. Elevation of sST2 levels has been reported in many other pathological conditions [68], meaning that ST2 inhibitors will have applications beyond aGVHD. IL-1 cytokine receptor [69] like ST2, contains an extracellular immunoglobulin-like and a cytosolic Toll/IL-1 receptor (TIR) domain. Targeting the extracellular domain (or ectodomain, ECD) with antibodies or biologics are proven therapeutic strategies and include anakinra [70] for IL1R and two ST2 antibody-based therapies, RG6149/AMG282 and GSK3772847, currently in phase 2 clinical trials [71]. Furthermore, small molecule ST2 inhibitors were discovered through a combination of High Throughput Screening, small angle X-ray scattering (SAXS) exploration, and computational analysis and proven active in vivo in two different aGVHD models [72]. Of note, ectodomains of cytokine receptors are challenging drug targets and no small molecule inhibitor has yet been reported to exhibit activity *in vivo* [71]. Lead compounds that we have discovered represent the first-in-class ST2 inhibitors exhibiting proof-of-concept of such inhibition in *in vivo* disease models. In contrast to biologics, ST2 small molecules inhibitors can be developed into orally bioavailable agents. They are also easier to administer and possess drug-like properties including efficient tissue penetration, modifiable pharmacological properties, half-lives, and have lower production costs. Inhibitor optimization to improve their potency, pharmacokinetic and pharmacodynamics properties is warranted.

VII Future research on biomarkers: from biomarkers to clinical trials

Given the progress being made in GVHD biomarker identification and validation, it is not surprising that clinical trial design have already begun incorporating biomarkers for some outcomes. Indeed, biomarker-based multicenter prospective study are currently done by the German group (see above, and Trial registration: ISRCTN03911524), and the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) evaluating sirolimus and prednisone in the low risk group calculated with an algorithm combining ST2 and REG3α (https://web.emmes.com/study/bmt2/protocol/1501_protocol/1501_protocol.html). There are also trials under development for patients with newly diagnosed aGVHD with high-risk biomarkers using intensified treatment. The German group is evaluating high risk for aGVHD early after their transplantation and use preemptive interventions. As with any screening test, improvements in sensitivity come at the expense of specificity and vice versa; which aspect to emphasize is a matter of clinical judgment. As discussed above, preliminary analysis showed that the clinical proteomics test measured at early time points (2–17 days after HSCT) was correlated with the future occurrence of aGVHD ≥ grade II with a

sensitivity of 87% and a specificity of 81%. Sensitivity and specificity remained high until day 30 post-HSCT, but then the pattern lost its predictive value at later time points. The toxicity of the intervention is an important consideration in trial design too, as excess toxicity from preemption will dampen acceptance of the strategy. A short course of corticosteroid therapy at the time during which markers of alloreactivity are increasing may be a reasonable therapy to test. The success of preemption must include a reduction not only in the incidence of aGVHD, but also in infectious complications and relapse. Future studies will be the establishment of clinical trials using both biomarkers for risk stratification and GVHD-specific drugs (as has been shown for ST2 in murine models [67, 72]) for treatment in high-risk populations.

VIII Conclusions

Clinical proteomics is a revolutionary field that identify and quantify proteins, molecules that are the most proximal to the real-time pathogenesis of alloreactivity as compared to genes and RNA. In a short time, the use of proteomics has led to the identification of novel biomarkers as well as mechanisms of allogeneic HSCT, which are unlikely to have been discovered by traditional hypothesis-driven research. A promising future approach is to use protein biomarkers in risk stratification to better employ current disease treatment modalities. Furthermore, the biomarker findings offer the potential for exploring targeted therapeutics as has recently been shown for ST2 inhibitors.

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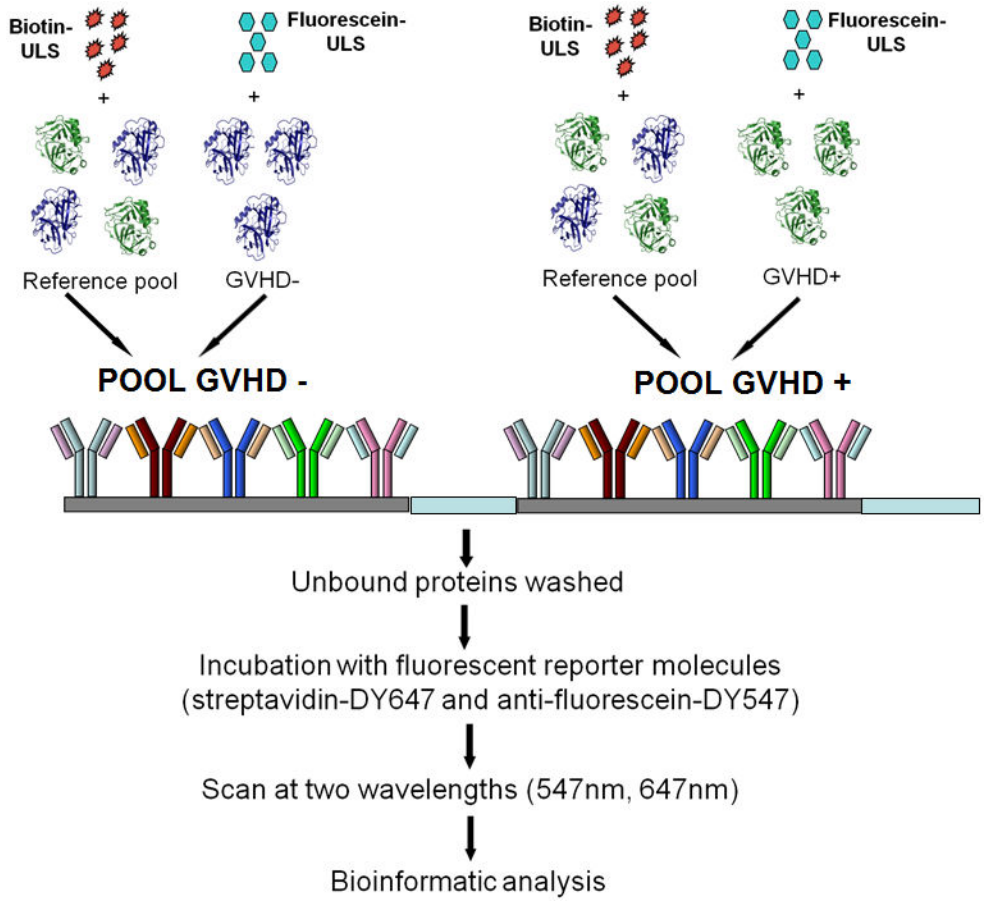

Figure 1.

Each of the GVHD+ (20 cases) and 21 GVHD− (20 controls) plasma samples were combined to form a reference pool, which was labeled with Biotin-ULS (in red). A second aliquot of each individual plasma sample was labeled with Fluorescein-ULS (in green). Sample B with fluorescein is a plasma from a patient without GVHD and Sample A with fluorescein is a plasma from a patient with GVHD. The labeled plasma proteins from one individual sample and one aliquot of the reference pool were hybridized to each side of the arrays overnight. After repeated washes, the arrays were probed with the fluorescent reporter molecules (streptavidin-DY647 and anti-fluorescein-DY547), washed, dried and scanned at two wavelengths within 24-48 hours before bioinformatic analysis.

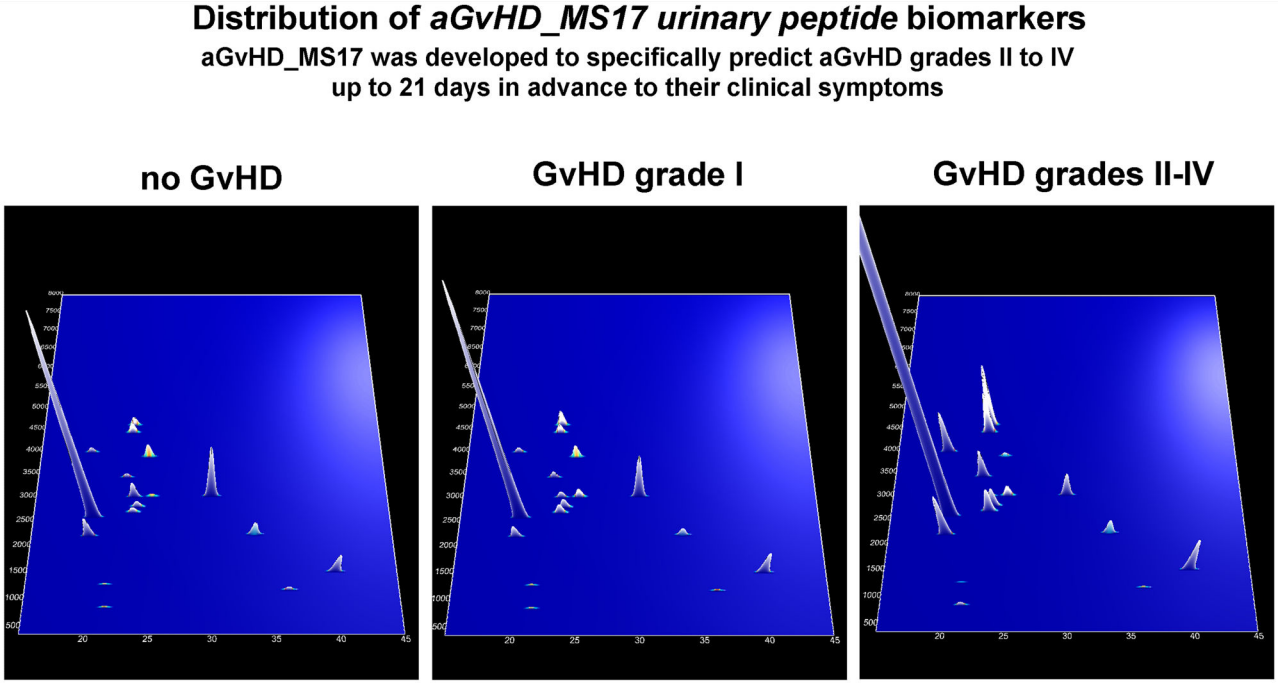

Figure 2.

Compiled capillary electrophoresis-mass spectrometry (CE-MS) spectra of naturally occurring urinary peptides included in the aGvHD_MS17 classifier for prediction of severe GvHD (grades II-IV) in leukemia patients after hematopoietic stem cell transplantation. Differences in peptide marker excretion are more prominent in GvHD II-IV than in no and grade I GvHD patient samples. CE migration time (x-axis, min) is plotted against log molecular weight (y-axis, kDa). Mean signal intensity is given as peak height.

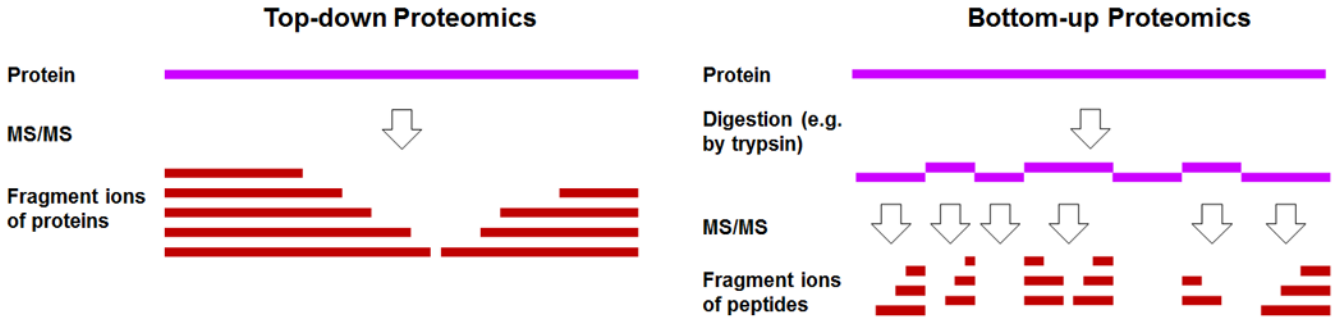

Figure 3.

Two tandem mass spectrometry (MS) proteomics strategies: (1) top-down proteomics which analyzes intact proteins, and (2) bottom-up proteomics which analyzes peptides in proteolytic digests. Computer algorithms then identify proteins based on peptide mass and fragmentation (MS/MS) information to search protein databases.

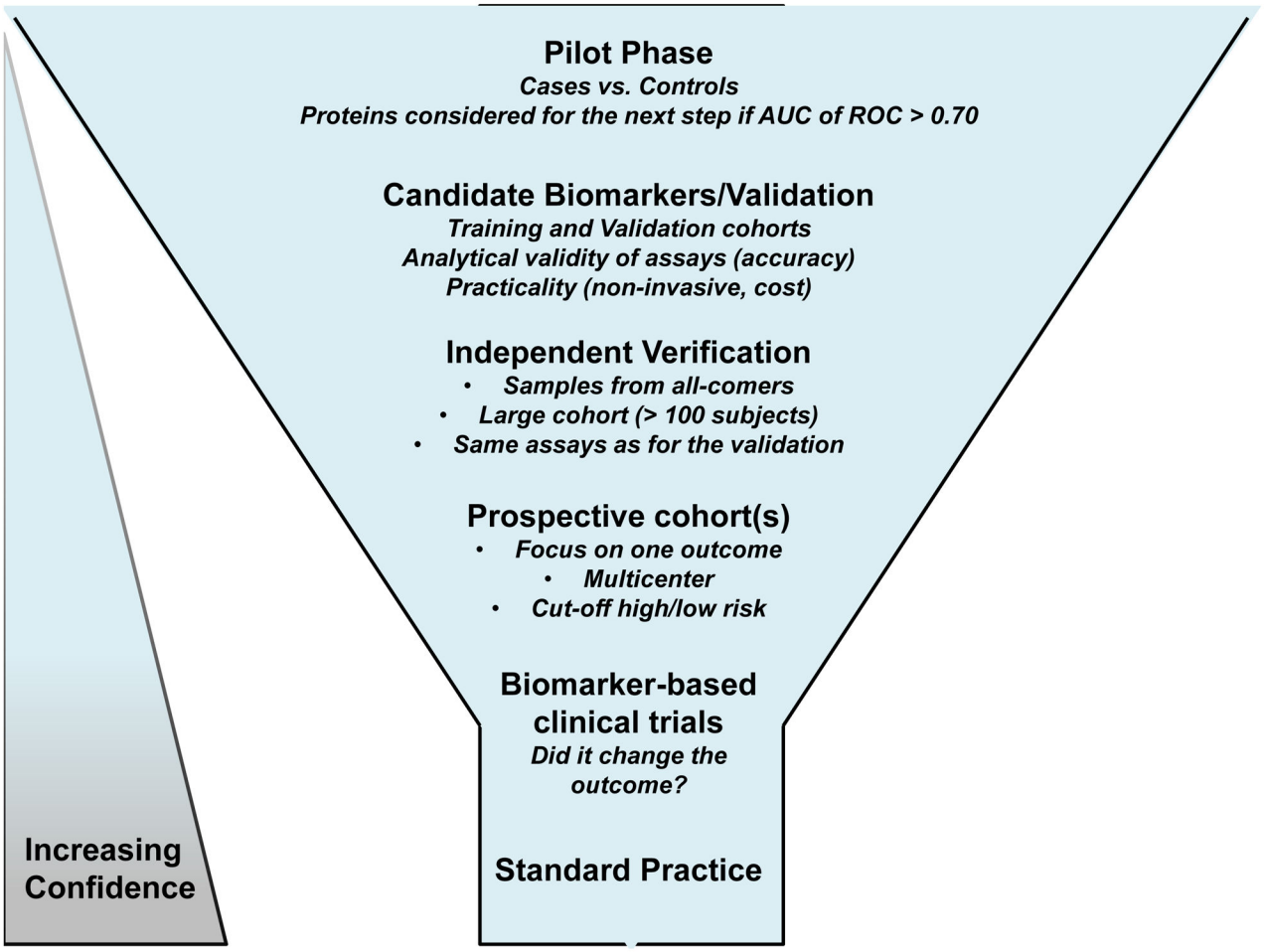

Figure 4.

Five major steps are required to develop a clinical test for standard practice. First, the pilot phase compares 20 to 40 cases and controls using discovery technologies. It is recommended that the term “candidate biomarker” be used to refer to findings of early phase studies when additional validation is needed. A protein will be considered a candidate marker worth to be pursued if the AUC of ROC > 0.70. Second, the validation phase is usually performed from a retrospective longitudinal case–control repositories with a training and validation sets. At this stage, the analytical validity of the tests and practicality are also evaluated. The third step focuses on few biomarkers using valid assay(s) and samples from a large independent verification cohort from all comers. The fourth phase requires a prospective multicenter validation with the goal to define cutoff for high and low risk for a specific outcome. It is hoped that this step will lead to a clinical test that will be approved by the U.S. Food and Drug Administration. Fifth, the biomarker can be used in a clinical trial and its impact on the reduction of disease burden on the population of interest is quantified. If the use of biomarker change the patients’ outcome, it is likely that it will be used in standard practice