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Biomarkers in graft versus host disease after allogeneic hematopoietic stem cell transplant

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BOSTON UNIVERSITY
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Thesis

**BIOMARKERS IN GRAFT VERSUS HOST DISEASE AFTER ALLOGENEIC
HEMATOPOIETIC STEM CELL TRANSPLANT**

by

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B.A., Colgate University, 2004

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HEMATOPOIETIC STEM CELL TRANSPLANT**

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ABSTRACT

Hematopoietic stem cell transplant was developed as a curative therapy to treat onco-hematological diseases (Schütt et al. 2007), and recently indications for this therapy have expanded to include solid tumors, hemoglobinopathies and other genetic diseases and disorders (Steward and Jarisch 2005). Two major types of hematopoietic stem cell transplant have been developed. Autologous transplants aim to deliver a massive dose of radiation and/or chemotherapy that is capable of ablating the hematopoietic stem cells in the bone marrow. The patient is then “rescued” from this lethal dose of treatment by an infusion of their own hematopoietic stem cells. Allogeneic transplants are designed to either functionally replace a cell class, or an enzyme or biological function absent in the patient, or to consolidate a remission in a onco-hematological disease via the graft-versus-tumor effect (Ofran and Ritz 2008). Two of the largest causes of non-relapse mortality from an allogeneic hematopoietic stem cell transplant are acute and chronic graft-versus-host disease, in which immune cells derived from the graft recognize normal host tissue as foreign and attack these tissues. A host of biomarkers for acute graft versus host disease have been identified, but there is almost none for chronic graft versus host disease. Herein, a methodology to discover and validate a biomarker(s) for the most common organ system affected by chronic graft versus host disease is proposed.

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LIST OF ABBREVIATIONS

GVHD.....	Graft-Versus-Host-Disease
HPC.....	Hematopoietic Progenitor Cell
LC-MS/MS..	High Performance Liquid Chromatography and Tandem Mass Spectrometry
miRNA.....	Micro Ribonucleic Acid
MS/MS.....	Tandem Mass Spectrometry
NCI.....	National Cancer Institute
NIH	National Institutes of Health
pH	Measure of Alkalinity or Acidity of a Solution
RNA.....	Ribonucleic Acid

INTRODUCTION

The first bone marrow transplants were born out of the US atomic energy program, as researchers strove to understand the mechanisms of radiation poisoning. Animal studies showed that radiation poisoning led to a failure of hematopoiesis and that cells from the spleen and bone marrow were capable of restoring hematopoiesis. E. Donnall Thomas performed and reported the first cases of syngeneic bone marrow transplants (Thomas et al. 1959).

Over the course of the intervening years hematopoietic cell transplantation has grown as a field, and the techniques, cell sources, conditioning, and indications for hematopoietic stem cell transplantation have grown from onco-hematological diseases such as Hodgkin's and non-Hodgkin's lymphomas (Schütt et al. 2007), and multiple myeloma (Ferland et al. 1998), to include many other diseases. Hematopoietic stem cell transplantation is now a standard of care for inherited metabolic storage disorders, severe autoimmune diseases, other genetic diseases (Steward and Jarisch 2005), as well as for some solid tumors, especially in the pediatric setting (Bochennek et al. 2012).

Two major types of hematopoietic stem cell transplants have been developed over the years. Traditionally, in both types of transplants, autologous (patients receive thawed autologous marrow or hematopoietic progenitor cells, apheresis), and allogeneic (patients generally receive fresh allogeneic stem cell-containing product) patients receive a

myeloablative conditioning regimen involving high dose total body irradiation and/or high dose chemotherapy agents (Bayraktar et al. 2013). In both cases, the doses of chemotherapy and/or radiation are considered lethal doses, as they destroy hematopoiesis in the patient.

Autologous transplants are indicated when clinicians wish to give a lethal dose of chemotherapy to ablate the bone marrow, and generally either clear the marrow of residual onco-hematological disease or to clear residual solid tumor (Femand et al. 1998). Allogeneic stem cell transplant, the second major type of hematopoietic stem cell transplant, was developed as clinicians sought to leverage the graft-versus-tumor effect to treat onco-hematological and other diseases. In this type of transplant, a donor is matched for several major human leukocyte antigens. In a modern transplant setting, at least 4 antigens are tested at a genetic level; there are three type 1 antigens, A, B, C, which present peptides from inside the cell to cytotoxic T lymphocytes, and one type 2 antigen, DR, which presents antigens from outside the cell to T-helper cells. Matching these genes at an allelic level decreases the risk of graft failure and decreases the risk of graft-versus-host disease (GVHD) (Morishima et al. 2002). The above-mentioned graft-versus-tumor effect is mediated by the interactions between donor cytotoxic T lymphocytes, donor natural killer cells and recipient specific cell surface allo-antigens and tumor specific antigens (Ofran and Ritz 2008).

Allogeneic hematopoietic stem cell transplants have been used to primarily treat onco-hematological diseases that respond poorly to treatment with chemotherapy agents.

Diseases such as acute myeloid leukemia (Clift et al. 1990), acute lymphoblastic leukemia (Terwey et al. 2013), chronic lymphocytic leukemia (Krejci et al. 2013), and chronic myeloid leukemia (Champlin et al. 2011) have been treated using allogeneic hematopoietic stem cell transplants with some success, increasing survival metrics for patients with these hard to treat diseases.

More recently, several transplant centers have pioneered new, non-myeloablative conditioning regimens allowing more patients (especially those over the age of 65) to undergo allogeneic hematopoietic stem cell transplants (Shimoni and Nagler 2002). These reduced intensity transplants for onco-hematological diseases strictly rely on the adaptive immunotherapy provided by the graft-versus-tumor effect (Ofran and Ritz 2008). The conditioning regimens for these transplants do not deliver an otherwise lethal dose of chemotherapy or radiation to the patient. Instead, they seek to clear some space in the bone marrow niche for the allogeneic hematopoietic stem cells to engraft, and avoid graft rejection by residual patient T cells (Servais, Baron, and Beguin 2011). These reduced intensity transplants are also seeing use in pediatric and adult settings to correct single gene mutations, such as sickle cell disease, thalassemia, and severe combined immunodeficiency (Steward and Jarisch 2005).

In addition to the growth of indications treatable with either autologous or allogeneic hematopoietic stem cell transplants, the number of cell sources has grown, each with advantages and disadvantages. The first cell source used in transplants were preparations of freshly harvested marrow from syngeneic twins (Thomas et al. 1959). Since then,

researchers have developed autologous transplants, which required the development of techniques from the field of cryobiology, allowing marrow to be cryopreserved and thawed safely, maintaining the integrity and function of cells (Berz et al. 2007). In addition to marrow, hematopoietic cells are now routinely harvested via their mobilization from the marrow to the blood with various agents (Mohty and Ho 2011) and leukapheresis (Körbling and Freireich 2011). These cells can be collected from the patient (autologous transplant) or from a donor (allogeneic). As previously mentioned, researchers have worked to determine the most important human leukocyte antigen alleles, not only allowing transplants from related donors, but also unrelated donors with the same HLA alleles as the recipient. To help identify potential unrelated donors, the National Marrow Donor Program was founded in 1986, in addition to other international registries also founded around this time. Finally, cord blood banking began in the late 1990's and has made transplants available for patients who are not well-represented in international transplant registries, but still require allogeneic hematopoietic stem cell transplants (Murphy et al. 2010).

Despite the growth of the field of hematopoietic stem cell transplantation, it is worth noting that it is not a trivial procedure and there are major complications and risks to this treatment. When treating onco-hematological diseases, there is always a risk of disease relapse. In many cases the hematopoietic stem cell transplant procedure is used as consolidation therapy, in an attempt to eradicate the last remaining neoplasm after the patient has achieved his or her best possible response to induction chemotherapy agents

(McCarthy and Hahn 2013). Relapse rates vary between diseases and within diseases by status and cytogenetics (Schütt et al. 2007). Myeloablative conditioning regimens lead to prolonged hematopoietic aplasia and immunologic suppression, leaving the patient at risk of developing opportunistic infections, requiring the transfusion of formed elements (such as platelets and red blood cells), granulocytes and pooled immunoglobulins. (Sorró et al. 2005). Depending on the cell source, cell dose and conditioning regimen, this period lasts, on average around 2-3 weeks.

If that was not enough, graft-versus-host disease, a dark reflection of the graft-versus-tumor effect, is a very substantial risk factor in allogeneic transplants. Briefly, despite the advances in human leukocyte antigen matching, and the development of prophylaxis measures, cells from the hematopoietic stem cell graft can recognize allo-antigens on host cells and attack the “foreign” cells and tissues. To complicate matters further, graft-versus-host disease is associated with an increased graft-versus-tumor effect and decreased risk of relapse (Storb et al. 2013). The causes and presentation of this disease are heterogeneous, which in the past, led to differences between treatment centers in the diagnosis and scoring of the severity of this disease. Studies conducted on GVHD prior to the establishment of consensus criteria can be confusing to interpret due to the various definitions of graft-versus-host disease (Filipovich et al. 2005). This confusion and lack of clarity in diagnostic standards lead to the development of the NIH consensus criteria (Filipovich et al. 2005). Briefly, the NIH consensus criteria defined two broad categories of GVHD, as well as a scoring system to help stratify risk and guide treatment:

The acute GVHD category is defined in the absence of diagnostic or distinctive features of chronic GVHD and includes (1) classic acute GVHD occurring within 100 days after transplantation and (2) persistent, recurrent, or late acute GVHD” (features of acute GVHD occurring beyond 100 days, often during withdrawal of immune suppression) (Filipovich et al. 2005).

The broad category of chronic GVHD includes (1) classic chronic GVHD (without features or characteristics of acute GVHD) and (2) an overlap syndrome in which diagnostic or distinctive features of chronic GVHD and acute GVHD appear together. It is currently recommended that systemic therapy be considered for patients who meet criteria for chronic GVHD of moderate to severe global severity (Filipovich et al. 2005).

Diagnosis of chronic GVHD is established by the presence of diagnostic and/or distinctive manifestations at any time post-transplant. To qualify as having classic chronic GVHD, a patient must either manifest at least one diagnostic clinical sign of chronic GVHD, or the patient must manifest at least one distinctive manifestation of chronic GVHD confirmed by biopsy or other relevant test (read biomarker) in the absence of acute GVHD (Filipovich et al. 2005). A diagnosis of overlap syndrome occurs when features of acute and chronic GVHD appear together. That is to say a patient either manifests at least one diagnostic clinical sign of chronic GVHD, or the patient must manifest at least one distinctive manifestation of chronic GVHD confirmed

by biopsy or other relevant test (read biomarker) at the same time as clinical features of acute GVHD present (Filipovich et al. 2005).

Diagnostic clinical signs and distinctive manifestations vary according to the organ affected. Skin chronic GVHD has many diagnostic manifestations, including distinctive features, as well as a number of common, non-distinctive manifestations that may belie the presence of skin chronic GVHD.

Diagnostic manifestations include poikiloderma (e.g., atrophic and pigmentary changes), lichen planus-like eruption (e.g., erythematous/violaceous flat-topped papules or plaques with or without surface reticulations or a silvery or shiny appearance on direct light), deep sclerotic features (e.g., smooth, waxy, indurated skin—“thickened or tight skin,” caused by deep and diffuse sclerosis over a wide area), morphea-like superficial sclerotic features (e.g., localized patchy areas of moveable smooth or shiny skin with a leathery-like consistency, often with dyspigmentation), or lichen sclerosus-like lesions (e.g., discrete to coalescent gray to white moveable papules or plaques, often with follicular plugs, with a shiny appearance and leathery consistency). Severe sclerotic features characterized by thickened, tight, and fragile skin are often associated with poor wound healing, inadequate lymphatic drainage, and skin ulcers from minor trauma (Filipovich et al. 2005).

Other tissues and organ systems generally have less diagnostic, distinctive and non-distinctive manifestations, including chronic GVHD of the lung, which has only one,

biopsy-proven diagnostic, bronchiolitis obliterans (Filipovich et al. 2005). Bronchiolitis obliterans in the setting of hematopoietic stem cell transplant is inevitably fatal unless the patient undergoes a lung transplant (Soubani et al. 2014).

As previously stated, it is difficult to diagnose chronic GVHD, because it is a diagnosis of exclusion. That is to say even in the presence of several diagnostic clinical signs or distinctive manifestations, other diseases and conditions that could lead to a similar clinical presentation must be ruled out due to a lack of definitive tests and biomarkers that indicate the presence or absence of the disease.

Acute GVHD has a higher rate of morbidity and mortality than chronic GVHD, and since the advent of better diagnostics and treatments for other peri-transplant complications, it has become the leading cause of non-relapse mortality during the first one hundred days post-transplant (Lee et al. 2013). Differentiation between late onset acute GVHD, acute GVHD, overlap syndrome and chronic GVHD in the NIH consensus criteria has been proven to be meaningful in terms of non-relapse mortality (Vigorito et al. 2009).

After diagnosis of chronic GVHD or overlap syndrome, the NIH consensus criteria and scoring system for individual organs and the global score may be used to assess the severity of the patient's disease and intervene appropriately (Filipovich et al. 2005). The scoring system has been validated for chronic GVHD, showing that the risk stratification of chronic GVHD in the NIH consensus criteria are meaningful in guiding treatment and predicting outcomes (Moon et al. 2014).

The consensus scoring system for individual organs, shown in Figure 1, allows for baseline and cross-sectional use and provides clinicians an easy method to acquire clinically relevant data regarding the extent and severity of disease (Filipovich et al. 2005). Each organ site of chronic GVHD is scored on a four-point scale, from 0, no symptoms present, to 3, which indicates severe impairment of the target organ sites (Filipovich et al. 2005). The number of organ sites and severity of each site involved determine the global score of chronic graft versus host severity. Mild chronic GVHD involves 1 to 2 organs (excluding the lungs) with a maximum score of 1 in all affected organs; moderate chronic GVHD involves (1) at least 1 organ or site with clinically significant but no major disability (maximum score of 2 in any affected organ or site) or (2) 3 or more organs or sites with no clinically significant functional impairment (maximum score of 1 in all affected organs or sites). A lung score of 1 will also be considered moderate chronic GVHD. Severe chronic GVHD indicates major disability caused by chronic GVHD (score of 3 in any organ or site). A lung score of 2 or greater will also be considered severe chronic GVHD (Filipovich et al. 2005). Moderate and severe chronic GVHD have been proven to be an indication for systemic corticosteroid treatment, and perhaps multiple agent treatment depending on the clinical situation (Moon et al. 2014).

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
PERFORMANCE SCORE: <input type="text"/> KPS ECOG LPS	<input type="checkbox"/> Asymptomatic and fully active (ECOG 0; KPS or LPS 100%)	<input type="checkbox"/> Symptomatic, fully ambulatory, restricted only in physically strenuous activity (ECOG 1, KPS or LPS 80-90%)	<input type="checkbox"/> Symptomatic, ambulatory, capable of self-care, >50% of waking hours out of bed (ECOG 2, KPS or LPS 60-70%)	<input type="checkbox"/> Symptomatic, limited self-care, >50% of waking hours in bed (ECOG 3-4, KPS or LPS <60%)
SKIN <u>Clinical features:</u> <input type="checkbox"/> Maculopapular rash <input type="checkbox"/> Lichen planus-like features <input type="checkbox"/> Papulosquamous lesions or ichthyosis <input type="checkbox"/> Hyperpigmentation <input type="checkbox"/> Hypopigmentation <input type="checkbox"/> Keratosis pilaris <input type="checkbox"/> Erythema <input type="checkbox"/> Erythroderma <input type="checkbox"/> Poikiloderma <input type="checkbox"/> Sclerotic features <input type="checkbox"/> Pruritus <input type="checkbox"/> Hair involvement <input type="checkbox"/> Nail involvement % BSA involved <input type="text"/>	<input type="checkbox"/> No Symptoms	<input type="checkbox"/> <18% BSA with disease signs but NO sclerotic features	<input type="checkbox"/> 19-50% BSA OR involvement with superficial sclerotic features "not hidebound" (able to pinch)	<input type="checkbox"/> >50% BSA OR deep sclerotic features "hidebound" (unable to pinch) OR impaired mobility, ulceration or severe pruritus
MOUTH	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Mild symptoms with disease signs but not limiting oral intake significantly	<input type="checkbox"/> Moderate symptoms with disease signs with partial limitation of oral intake	<input type="checkbox"/> Severe symptoms with disease signs on examination with major limitation of oral intake
EYES Mean tear test (mm): <input type="checkbox"/> >10 <input type="checkbox"/> 6-10 <input type="checkbox"/> ≤5 <input type="checkbox"/> Not done	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Mild dry eye symptoms not affecting ADL (requiring eyedrops ≤ 3 x per day) OR asymptomatic signs of keratoconjunctivitis sicca	<input type="checkbox"/> Moderate dry eye symptoms partially affecting ADL (requiring drops > 3 x per day or punctal plugs), WITHOUT vision impairment	<input type="checkbox"/> Severe dry eye symptoms significantly affecting ADL (special eyewear to relieve pain) OR unable to work because of ocular symptoms OR loss of vision caused by keratoconjunctivitis sicca
GI TRACT	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Symptoms such as dysphagia, anorexia, nausea, vomiting, abdominal pain or diarrhea without significant weight loss (<5%)	<input type="checkbox"/> Symptoms associated with mild to moderate weight loss (5-15%)	<input type="checkbox"/> Symptoms associated with significant weight loss >15%, requires nutritional supplement for most caloric needs OR esophageal dilation
LIVER	<input type="checkbox"/> Normal LFT	<input type="checkbox"/> Elevated Bilirubin, AP*, AST or ALT <2 x ULN	<input type="checkbox"/> Bilirubin >3 mg/dl or Bilirubin, enzymes 2-5 x ULN	<input type="checkbox"/> Bilirubin or enzymes > 5 x ULN

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
LUNGS[†]	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Mild symptoms (shortness of breath after climbing one flight of steps)	<input type="checkbox"/> Moderate symptoms (shortness of breath after walking on flat ground)	<input type="checkbox"/> Severe symptoms (shortness of breath at rest; requiring O ₂)
FEV1 <input type="text"/>				
DLCO <input type="text"/>	<input type="checkbox"/> FEV1 > 80% OR LFS=2	<input type="checkbox"/> FEV1 60-79% OR LFS 3-5	<input type="checkbox"/> FEV1 40-59% OR LFS 6-9	<input type="checkbox"/> FEV1 ≤39% OR LFS 10-12
JOINTS AND FASCIA	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Mild tightness of arms or legs, normal or mild decreased range of motion (ROM) AND not affecting ADL	<input type="checkbox"/> Tightness of arms or legs OR joint contractures, erythema thought due to fasciitis, moderate decrease ROM AND mild to moderate limitation of ADL	<input type="checkbox"/> Contractures WITH significant decrease of ROM AND significant limitation of ADL (unable to tie shoes, button shirts, dress self etc.)
GENITAL TRACT	<input type="checkbox"/> No symptoms	<input type="checkbox"/> Symptomatic with mild signs on exam AND no effect on coitus and minimal discomfort with gynecologic exam	<input type="checkbox"/> Symptomatic with moderate signs on exam AND with mild dyspareunia or discomfort with gynecologic exam	<input type="checkbox"/> Symptomatic WITH advanced signs (stricture, labial agglutination or severe ulceration) AND severe pain with coitus or inability to insert vaginal speculum
Other indicators, clinical manifestations or complications related to chronic GVHD (check all that apply and assign a score to its severity (0-3) based on its functional impact where applicable (none – 0, mild -1, moderate -2, severe – 3))				
Esophageal stricture or web ___	Pericardial Effusion ___	Pleural Effusion(s) ___		
Ascites (serositis) ___	Nephrotic syndrome ___	Peripheral Neuropathy ___		
M yasthenia Gravis ___	Cardiomyopathy ___	Eosinophilia > 500µl ___		
Polymyositis ___	Cardiac conduction defects ___	Coronary artery involvement ___		
Platelets <100,000/µl ___	Progressive onset ___			
OTHERS: Specify: _____				

Figure 1: Organ scoring of chronic graft versus host disease. Adapted from Filipovich et al. 2005.

Chronic GVHD contributes significantly to late (>100 days post transplants) non-relapse mortality, as shown Table 1 and Figure 2 (Pidala et al. 2011; Socié et al. 1999).

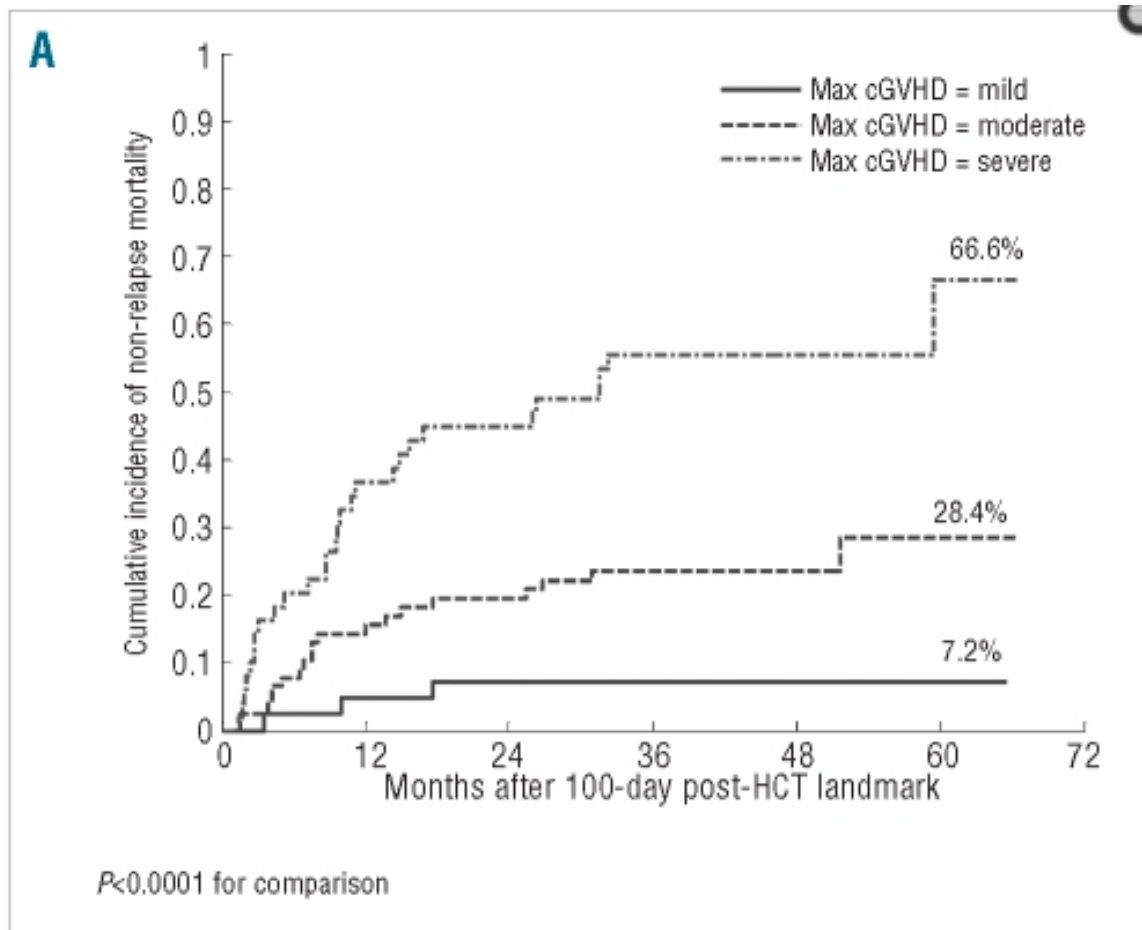


Figure 2: Non-relapse mortality according chronic graft versus host disease severity. Cumulative incidence of non-relapse mortality (y-axis) plotted against time in months after 100 days post-transplant landmark. Grades of chronic graft versus host disease are given according to NIH consensus standard. Higher grades significantly associated with a greater risk of non-relapse mortality. 244 patients were included in this study. Adapted from Pidala et al. 2011.

Table 1: Primary causes of death among patients who were disease-free two years after transplantation. The burden of chronic graft versus host disease on long term survivors after an allogeneic hematopoietic stem cell transplant. In long-term survivors, 30% of deaths are attributable to graft versus host disease. Adapted from Socié et al. 1999.

CAUSE OF DEATH	AML	ALL	CML	APLASTIC ANEMIA	TOTAL
	(N=214)	(N=167)	(N=238)	(N=60)	(N=679)
	number of patients (percent)				
Relapse	117 (56)	79 (48)	108 (47)	0	304 (46)
GVHD	47 (23)	38 (23)	81 (36)	38 (66)	204 (31)
Infection without GVHD	11 (5)	7 (4)	14 (6)	7 (12)	39 (6)
Bacterial	5	2	5	3	15
Viral	2	1	3	2	8
Fungal	0	1	0	0	1
Protozoal	0	1	0	0	1
Infectious pneumonia†	2	0	4	1	7
Other infection†	2	2	2	1	7
New cancer	15 (7)	16 (10)	8 (4)	1 (2)	40 (6)
Organ failure	11 (5)	14 (9)	10 (4)	5 (9)	40 (6)
Other‡	7 (3)	10 (6)	7 (3)	7 (12)	31 (5)
Unknown	6	3	10	2	21

*Percentages shown are of deaths with known causes. Because of rounding, not all percentages total 100.

†The type of infection was not otherwise specified.

‡Other causes of death were hemorrhage in 10 patients (3, 1, 3, and 3 in patients with AML, ALL, CML, and aplastic anemia, respectively), interstitial pneumonitis in 6 patients (3, 1, and 2 in patients with ALL, CML, and aplastic anemia, respectively), drug reaction in 1 patient with aplastic anemia, and miscellaneous causes in 14 patients (4, 6, 3, and 1 in patients with AML, ALL, CML, and aplastic anemia, respectively).

Even using the NIH consensus criteria, diagnosis of the GVHD is complicated, and risky. Many of the presentations and symptoms of GVHD are similar to other peri-

transplant and post-transplant diagnoses. In some cases initiation of front line treatment for any type of GVHD, e.g. systemic corticosteroids, can be lethal if misdiagnosed (Filipovich et al. 2005). Currently all types of GVHD are diagnosed by the presence or absence of symptoms, and confirmed with a biopsy of the affected tissues and organ system (Coron et al. 2014). The sensitivity and specificity of this gold standard is below that of many other tests indicating certain disease conditions and the development of superior tools for diagnosis and confirmation of GVHD are required.

As previously stated, and illustrated in Figure 1, chronic GVHD manifests in several target organs and tissues, including the skin, mouth, liver, lung, eye, fascia, gastrointestinal tract and genitals (Jacobsohn et al. 2012). More than one tissue or organ may be affected by chronic GVHD. It may co-present as an overlap syndrome in which diagnostic features of acute and chronic GVHD appear together (Filipovich et al. 2005). Recently some biomarkers have been identified for acute GVHD and are being tested in clinical trials in many hematopoietic stem cell transplant centers. Suppressor of Tumorigenesis 2 (ST2) was recently developed and has been validated to diagnose acute GVHD and can predict transplant related mortality (Ponce et al. 2014) However only one biomarker has been validated for chronic GVHD (Devic et al. 2014), so there is a great need for biomarkers to distinguish chronic GVHD from other complications (Paczesny et al. 2013).

PUBLISHED STUDIES

Now that GVHD has been defined and the need for biomarkers to identify chronic GVHD has been described, some candidate biomarkers for this disease will be enumerated. Simply stated a biomarker is a biological molecule found in body fluids and/or tissues that signifies the presence or absence of a normal or abnormal process, condition or disease. It may be used to determine how the body will respond to treatment and may be used to guide risk stratification and treatment (“Definition of Biomarker - NCI Dictionary of Cancer Terms” 2015). A biomarker can be a specific protein or enzyme found in the blood, urine or tissue, a specific circulating cell type (identified by cluster of differentiation markers as assayed by flow cytometry), or a type of ribonucleic acid, e.g. micro RNA, which can also be found in body fluid. Some also consider pre-transplant risk factors, such as the presence or absence of certain alleles in the recipient and/or donor, biomarkers; however, while these factors are important and worthwhile, they do not meet the later part of the standard definition of a biomarker (“Definition of Biomarker - NCI Dictionary of Cancer Terms” 2015).

Cellular markers are of great interest in studying this particular disease, because immune cells expressing cluster of differentiation markers are the effector cells which are responsible for causing many of the symptoms and damage found in GVHD. Several studies have examined the relative quantity of regulatory T cells, regulatory B cells, natural killer, cytotoxic T lymphocytes, and T helper subsets at various time points after transplant. Greinix et al., have recently identified several populations of cells that

independently predict future onset of chronic GVHD in a discovery set (Greinix et al. 2014; Kuzmina et al. 2011). Sarantopoulos et al. have shown that B-cell activating factor and immature, or arrested, B cell phenotypes are correlated with active chronic GVHD (Sarantopoulos et al. 2007). Finally, other groups have isolated regulatory T cells and analyzed the transcriptomes of these cells seeking to identify biomarkers that could be used both in the diagnosis of acute and chronic GVHD and to potentially identify response-to-treatment (Ukena et al. 2012). It is worth noting that none of the candidate biomarkers identified by this body of literature have been validated.

Micro ribonucleic acids (miRNA) are small, non-coding sequences of RNA that silence messenger RNA and post-transcriptionally regulate expression of genes. miRNAs are present in blood, and patterns of miRNA expression are indicative of normal function or pathology, including autoimmune diseases, such as systematic lupus erythematosus (Atarod and Dickinson 2013; Xie et al. 2014). The technology for analyzing miRNAs lags behind both proteomics and analysis of cellular markers using flow cytometry techniques, and has yet to yield many candidate biomarkers for either acute or chronic GVHD (Paczesny et al. 2013).

There are many types of potential biomarkers that can be found in various tissues and fluids. Molecular techniques allow scientists to assay the concentrations of different proteins found in body fluids. The field is well developed and there are a variety of tools researchers can use to identify potential biomarkers. Researchers can select and detect levels of various proteins in serum or blood samples using either enzyme-linked

immunoabsorbent assays or multiplexed immunoassays in search of a biomarker for their disease of interest. However, there are problems with this approach, including selection bias, for example, selecting cytokines and soluble cytokine receptors, rather than markers of tissue specific damage (Berger et al. 2013)

In addition to selection bias, there are potential pitfalls that may arise with the molecular techniques used, including cross reactivity (Keustermans et al. 2013). In this situation, cross reactivity may be defined as a substance within the sample that raises or lowers the determined concentration of the protein from the true concentration of the protein in the sample. In addition to this factor, factors affecting sample quality, including the anticoagulant used (if blood), the time from collection to processing (urine, serum, plasma), the length of time in storage at -80°C (serum and plasma), and the number of “freeze-thaws” specimens undergo can radically change the results obtained via these molecular methods (Parkitny et al. 2013; Keustermans et al. 2013). Despite the pitfalls, there are significant advantages that can be leveraged using molecular techniques to identify and detect biomarkers. Many hematopoietic transplant centers keep an archive of samples collected at various timepoints from patients undergoing transplant procedures. Combining the readily accessible number of samples available and these techniques can lead to a study with great statistical power that can look at the levels of specific candidate biomarkers at various time points easily (Paczesny 2013).

Proteomics, the study of all of the proteins expressed by a specific cell, tissue, organ or organism is a powerful tool to screen potential biomarkers. Mass spectrometry and

high performance liquid chromatography have been used to detect, with absolute specificity, the identity and concentration of proteins in two samples representing two experimental conditions (Faca et al. 2006). There are two general workflows that have been used in biomarker identification in transplantation and both usually leverage a similar method to quantify protein concentration, i.e., liquid chromatography tandem mass spectrometry (LC MS/MS) (Faca et al. 2006), but get to this step in different ways. These workflows are known as top-down and bottom-up, or shotgun proteomics.

Bottom-up proteomics workflows are more established, but present some disadvantages when used to identify biomarkers (Parks et al. 2007). Briefly, bottom-up proteomics work flows involve the digestion of complex protein mixtures using trypsin, yielding a peptide mixture that is generally labeled with isobaric tags (e.g., condition A is labeled with heavy isotopes of carbon and nitrogen and condition B is labeled with light isotopes) prior to separation and analysis (Han, Aslanian, and Yates 2008). During analysis the relative concentration and identity of proteins in the sample is determined via an algorithm based on the peptides detected by the mass spectrometer (Han, Aslanian, and Yates 2008).

Top-down proteomics eliminates the upfront digestion of proteins in a sample into a peptide mixture. Instead it relies on labeling proteins in a sample with isobaric tags before upfront fractionation of the sample using liquid chromatography. After the undigested proteins have been tagged, they are ionized in the gas phase. The ionized, intact proteins are assayed by mass spectrometry, then are directly fragmented and inside

the mass spectrometer by electron-capture dissociation or electron-transfer dissociation and re-analyzed to determine the identity and concentration of proteins with absolute specificity (Han, Aslanian, and Yates 2008).

Originally bottom-up approaches were favored in biomarker identification because of the wide dynamic range of analyses, and robustness of the methodology. Key work by Park and Han led to an increase in the dynamic range and usability of top-down approaches (Han et al. 2006; Parks et al. 2007). This, coupled with improvements in algorithms used to identify proteins given the MS/MS spectra, and the ability of top-down proteomics work flow to identify proteins and quantify them absolutely in two different experimental conditions, to identify post-translation modifications, and to identify smaller proteins, under 30 kilodaltons (proteins this small may not have trypsin cleavage sites), that may be biologically relevant, makes top-down proteomics superior for biomarker discovery (Parks et al. 2007). However, depending on sample source, institutional expertise and equipment, bottom-up approaches can be utilized as well (Devic et al. 2014).

When using top-down or bottom-up proteomic analyses to identify potential biomarkers for a disease condition, the required power of the study, thus the number of samples required, is an order of magnitude lower than required when using immunoassays. Between 10 and 20 unique samples collected from control patients and patients exhibiting the disease condition are required for analysis (Paczesny 2013). The sample can be serum, plasma or other body fluids (Devic et al. 2014). As discussed, the

selection, preparation and storage are critical to ensuring that the experimental results, i.e. presence/absence, concentration, reflect the presence or absence of the disease state as opposed to effects introduced by poor handling (Keustermans et al. 2013). Once the mass spectra are analyzed, there are generally between 10-200 proteins that differ in concentration greater than two-fold between the two conditions (Ferrara et al. 2011).

Researchers choose targets for validation based on biological activity, availability of commercially available antibodies, and preferential expression in the target organ(s), if applicable (Hanash, Pitteri, and Faca 2008). The validation set (hundreds of patients and samples), is then analyzed with ELISA or multiplex studies (Ferrara et al. 2011). The validation set should include patients from multiple transplant centers, with samples stored under standard and consistent conditions. After validation of the biomarker, additional prospective studies must be developed to determine the utility of this biomarker in guiding clinical decisions and interventions.

Each technique discussed above, miRNA, flow cytometry, and proteomic analysis has advantages and disadvantages. The most logical and established method of biomarker discovery involves LC-MS/MS screening using top-down or bottom-up methodology followed by validation using standard immunological methods (Paczesny 2013). Not only is this methodology robust, with a history of delivering results, but it has a lower cost and technical barrier to entry than some of the newer techniques used to identify biomarkers. However, the costs of performing these studies are not trivial.

Assuming adequate resources are available, there remain recurring and additional concerns. The acquisition, processing and storage of samples are critical and must be appropriately managed for both the mass spectrometry analysis to yield potential targets for validation and for follow-up validation studies and clinical trials. Multiple studies have shown that the preparation of samples prior to processing and storage conditions can affect the measured concentration of cytokines and other volatiles (Chaturvedi et al. 2011; Parkitny et al. 2013; Keustermans et al. 2013) as measured by either single or multiplexed immunoplex assays. In addition to concerns regarding the samples used in a study there are additional concerns that must be considered when designing all phases of a biomarker discovery study.

As discussed in the introduction there are a host of underlying diseases that can be treated with allogeneic hematopoietic stem cell transplant, and a variety of conditioning regimens and GVHD prophylaxis strategies used among transplant centers. There are also at least three cell sources for allogeneic transplants, including HPC, Apheresis, HPC, Marrow and HPC, Cord Blood. These source materials can be manipulated in vivo or ex vivo to functionally alter a population, i.e. enrich or reduce specific populations of cells (Marek et al. 2014). In addition to all the confounding factors listed above, transplant patients tend to be of somewhat advanced age, especially in the adult setting; therefore it may be worthwhile to eliminate this confounding variable by running validation studies of the candidate biomarker with age-matched, disease-free patient controls (Devic et al. 2014).

After identifying a candidate biomarker, and dealing with the above-mentioned confounding variables and concerns relevant to experimental design, the validation set provides the first chance to determine the potential clinical usefulness of the candidate biomarker. Ideally, a biomarker should have predictive properties, with some sensitivity and specificity, regarding whether or not the disease in question will manifest. If a biomarker passes this test, researchers must design further clinical trials to evaluate the use of the biomarker or panel in order to determine whether or not it can stratify risk (Paczesny et al. 2009), guide interventions (Paczesny et al. 2013), or perform in different conditions than demonstrated in the discovery or validation set (Paczesny et al. 2010; Ponce et al. 2014). Ideally, a biomarker that speaks to the heart of a disease process should be able to perform in multiple centers in various settings (Addona et al. 2009).

Despite the concerns and difficulties in biomarker discovery, of which there are many, biomarker panels must be developed for chronic GVHD. That is to say that various serum/plasma, and potentially urine and saliva, biomarkers must be found for the various manifestations of chronic GVHD. The best bet for identifying biomarkers in these fluids is to leverage mature discovery technologies, such as LC-MS/MS proteomics workflows to search for proteins with differing concentrations in the setting of classic chronic GVHD.

After identification of likely 10-100s of protein concentrations that vary at least two-fold in the disease state as compared to non-disease controls, a researcher with clinical experience, knowledge of immunology and the natural history and pathogenesis of

chronic GVHD must select one or more potential biomarkers for validation (Paczesny et al. 2009). Ideally there will already be a developed and well-characterized antibody for the candidate biomarkers that can be used in either singlet or multiplexed immunoassays (Rozmus and Schultz 2011). Validation studies must prove that the biomarker can predict the development of clinical signs and symptoms of the disease, and ideally, potential responsiveness to treatment, although this is largely a goal for further clinical trials.

The forthcoming proposal for research will discuss in more detail the target organs in chronic GVHD that will be mined for biomarkers. It will also determine the appropriate sample source to assay for each target organ as well as the development of a discovery set. In addition it will explain, in more detail, the proteomics tool best suited to analyze the discovery set, i.e., the intact protein analysis system, which has been used in various biomarker discovery experiments in acute and chronic GVHD (Ferrara et al. 2011; Paczesny et al. 2010; Devic et al. 2014). Appropriate experiments to validate the enhanced expression or down-regulation of candidate biomarkers in the tissue of interest must be performed, then a larger validation set should be used to determine the specificity and sensitivity of the biomarker in determining the presence of organ-specific chronic GVHD. In addition, this validation set can be tested to statistically determine if concentrations of the biomarker can predict response to treatment and stratify risk, independently of pre-existing conditions and independent of NIH consensus criteria scores of chronic GVHD (Paczesny et al. 2010). After these experiments are completed,

further clinical trials can be designed to test the the biomarker in specific transplant conditions and populations, and again examine, this time a priori, the significance and clinical utility of the biomarker, to determine if changing clinical practice based on biomarker signal can help reduce morbidity and mortality of organ-specific chronic GVHD.

PROPOSAL

A number of studies have demonstrated that researchers are able to identify candidate biomarkers from saliva (Devic et al. 2014), urine (Beretov et al. 2014), skin (Paczesny et al. 2010), and blood (Paczesny et al. 2009). Since chronic GVHD does not manifest principally in one anatomic area, and generally presents in multiple target organs, it seems that the most useful source of sample for the discovery experiment should be either blood, skin, liver, lungs, or mucosa (Jacobsohn et al. 2012). The sample tissue/fluid for the discovery set can be further parsed arrow down our by considering the relative impact of the different types of chronic GVHD, and the current candidate biomarkers being evaluated for chronic GVHD.

Recently, Devic published a paper identifying candidate biomarkers for oral / gastrointestinal (mucosal) chronic GVHD (Devic et al. 2014). These candidate biomarkers , IL-1 receptor antagonist and cystatin B, showed decreased expression in patients with active oral chronic graft-versus-host disease and were validated using enzyme-linked immunosorbant assays (Devic et al. 2014). These recent findings suggest that we should look elsewhere for a candidate biomarker in order to give clinicians biomarkers for other end organ chronic GVHD.

The skin is the most common location of chronic GVHD and has a high hazard ratio indicating it contributes significantly to non-relapse morbidity and mortality(Wingard et al. 1989; Goerner et al. 2002). Examples of clinical manifestation of skin chronic GVHD

are shown in Figure 3. Common manifestations include cutaneous and sclerotic pathology (Jacobsohn et al. 2012).

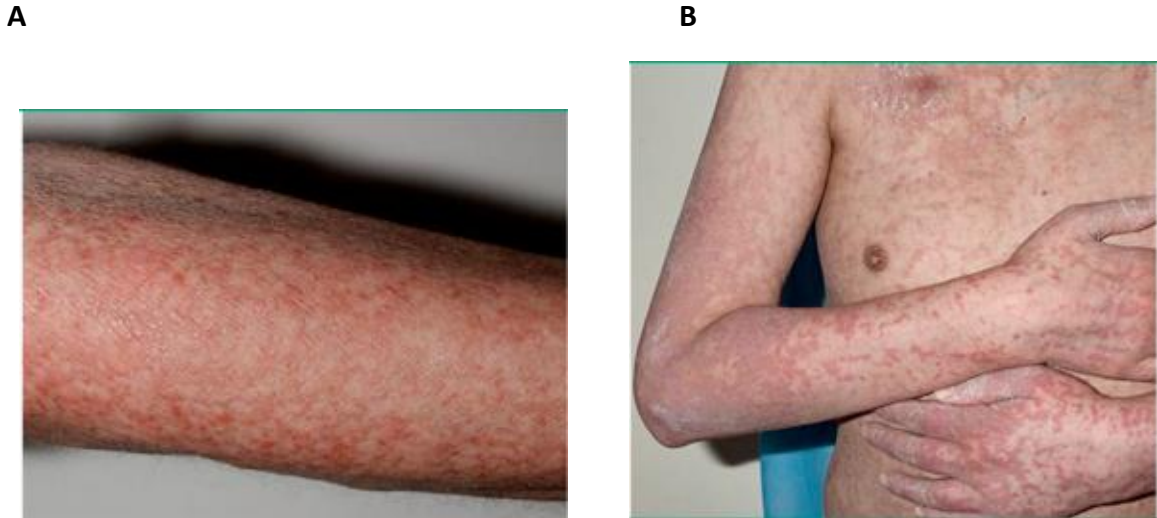


Figure 3: Clinical manifestation of cutaneous and sclerotic chronic graft versus host disease. (A) Polkioderma - Polkiodermatous changes, mottled pigmentation and erythema present. **(B)** Cutaneous – Lichen-planus like pathology, papules and plaques present on trunk and extremities. Adapted from (Cohen 2015)

A recent study has validated the NIH composite 0-3 score in patients with cutaneous skin chronic GVHD, dismissing the clinical utility of most other scales. The study assessed the ability of a number of clinical tools to predict non-relapse mortality, and physician and patient perception of skin chronic GVHD progression. The NIH composite 0-3 score considers the extent of skin involvement, symptoms and presence of sclerotic features. A score of 0 means that the patient is asymptomatic. A score of 1 indicates the absence of sclerotic features and body surface area coverage of less than 18%. A score of 2 indicates either the presence of sclerotic features or 19-50% body surface area coverage. Finally a score of 3 indicates either greater than 50% body surface area coverage, or deep

sclerotic features, or impaired mobility, ulceration or severe pruritis (Filipovich et al. 2005). The study found a strong correlation between the score and non-relapse mortality, shown in Figure 4 (Jacobsohn et al. 2012). Other groups have identified acute GVHD markers in the skin, indicating that it is feasible to search for tissue specific markers in the plasma or serum of patients (Paczesny et al. 2010).

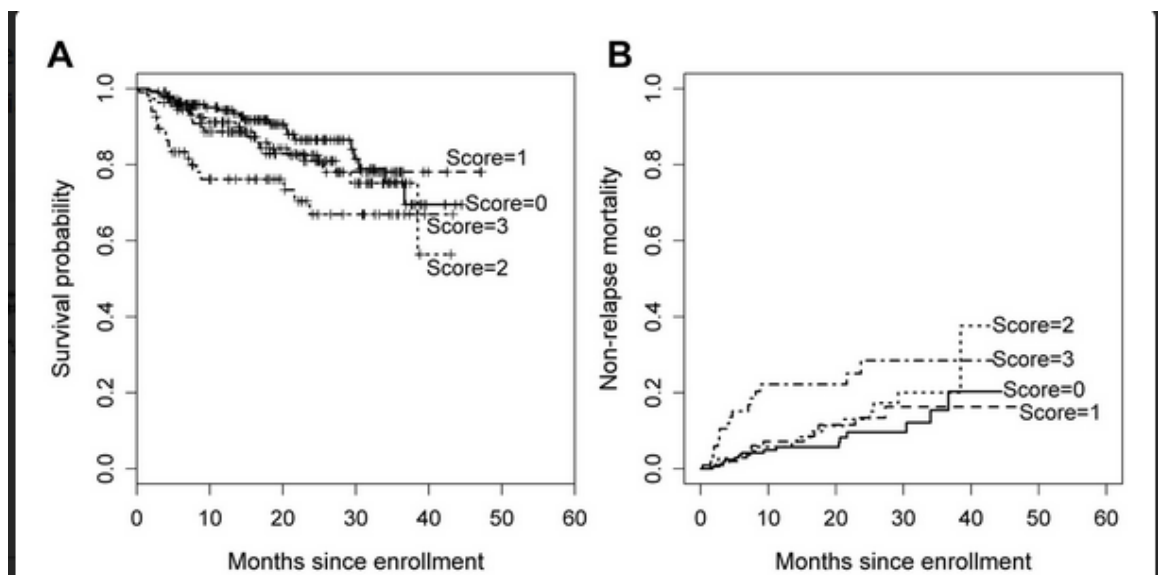


Figure 4: Overall survival and non-relapse mortality by NIH composite 0-3 score skin chronic graft versus host disease. 458 patients enrolled in this study to assess the ability of various clinical instruments to predict overall survival and non-relapse mortality in skin manifestations of chronic graft versus host disease. (A) Overall survival (y-axis) plotted against months since enrollment in study, each curve represents patients score at baseline. Grade 3 significantly associated with higher mortality and non-relapse mortality. (B) Cumulative incidence of non-relapse mortality (y-axis) plotted against months since enrollment in study. Adapted from (Jacobsohn et al. 2012).

Chronic GVHD also manifests commonly in the lungs, as previously stated, with up to 50% of patients who develop chronic GVHD exhibiting obstructive or restrictive

changes (Jacobsohn et al. 2012). Long term survivors of allogeneic hematopoietic stem cell transplant exhibiting pulmonary dysfunction, and full-blown bronchiolitis obliterans have a 15.1-fold increased risk of late mortality when compared to the general population (Bhatia et al. 2007). Bonafide lung chronic GVHD, biopsy-proven bronchiolitis obliterans affects about 6% of allogeneic hematopoietic stem cell transplant patients, but has a five year survival of 13% (Williams et al. 2009), slightly lower than the general diagnosis of severe chronic GVHD at 15% (S. J. Lee and Flowers 2008). As previously stated, many patients show clinical signs and symptoms of pulmonary dysfunction, however only a small portion of this subset of patients develops either type of bronchiolitis obliterans. Because of this disparity, all patients who have undergone allogeneic transplant should be screened according to the diagnostic workup and clinical care algorithm similar to the recommendations of Hildebrandt, shown in Figure 5 (Hildebrandt et al. 2011).

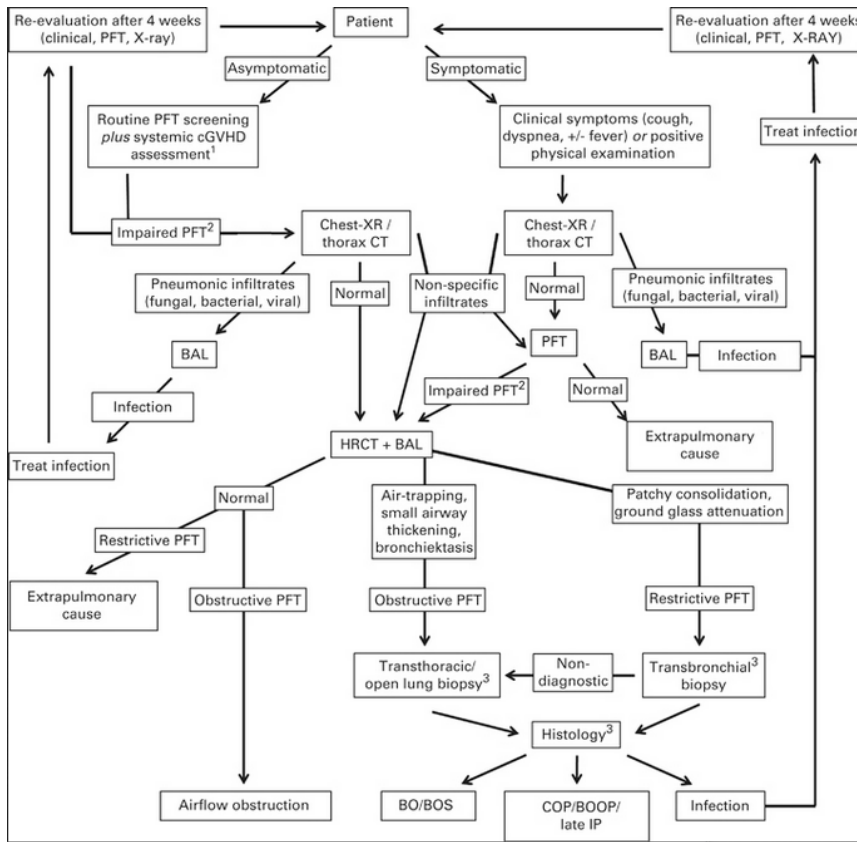


Figure 5: Diagnosis, work up and treatment of lung injury following allogeneic hematopoietic stem cell transplant. Pulmonary function test screening is recommended 3, 6, 9, 12, 18 and 24 months after allogeneic hematopoietic stem cell transplant and then once per year. Clinical decisions are made based on radiologic and pathology samples taken from lung lavage samples, which are not trivial to obtain. Adapted from (Hildebrandt et al. 2011).

Incorrect diagnosis of bronchiolitis obliterans, and the initiation of inhaled or systemic corticosteroids in the setting of infection, must be avoided in this setting. A plasma biomarker could greatly aid this workflow and help guide decision-making at any of the bifurcation points found in the clinical workflow. For example, a patient with non-specific infiltrates discovered by computed tomography x-ray scan and impaired pulmonary function tests requires a lung biopsy in order to move forward with diagnosis and treatment. Today, in cases where it is clinically difficult to obtain a biopsy, clinicians

must treat empirically. A biomarker can help better clinical decision making at this key point and lead to better clinical outcomes (Paczesny 2013). A biomarker might also be able to show which patients respond to front-line treatment for newly diagnosed airflow obstruction and decreased pulmonary function tests after allogeneic stem cell transplant. A clinical marker that could show patient response to treatment quickly could be used to modify clinical workflows – perhaps adding systemic to localized inhaled steroids in an effort to decrease plasma cell-mediated disposition of immunoglobins (Sarantopoulos et al. 2015).

Now that the two organ targets of GVHD have been identified in order to search for biomarkers for skin and lung manifestations chronic GVHD, a discovery set must be acquired. Depending on the strength of the treatment center's general hematopoietic stem cell transplant sample collection protocol, the researcher should be able to identify approximately 10 heparinized plasma samples from patients who have undergone allogeneic hematopoietic stem cell transplants without developing either type of GVHD; these samples will serve as the control group. The researcher should also be able to identify 10 heparinized plasma samples for patients who have developed either single organ system isolated, biopsy-proven severe chronic GVHD of either the skin or lung, in which case 5 samples from each condition should be utilized, or samples from patients exhibiting both pathologies may be used.

One factor that should be controlled for is the sample quality, that is to say the length of time in storage, and number of freeze-thaws from -80°C (Keustermans et al. 2013).

Other factors that should be considered are the intensity of the conditioning regimen (myeloablative or non-myeloablative), patient age, and the number of unrelated donors in each group (Paczesny et al. 2010). In particular, the control group needs to be matched for pre-transplant lung infections, including colonization with *Candida* and other fungi, as well as bacterial infections in patients with severe lung chronic GVHD, so that the study does not conflate markers of bacterial and fungal damage to the lungs as being a marker of B cell-mediated chronic GVHD. It also makes sense to control for the cell source and relevant manipulations. Indication for transplant should not be a concern.

Once the discovery set has been established, and the appropriate samples identified, they should be analyzed using a proteomic discovery tool. A top-down proteomics workflow has some advantages when compared to an easier to execute bottom-up, or shotgun proteomics approach. Top-down proteomics workflows have the advantage of being able to identify and discriminate between various isoforms of proteins, but are technically more complex than bottom-up approaches, and have difficulty identifying larger proteins (Han et al. 2006). There is a workflow that relies on extensive fractionation of the starting sample called intact-protein analysis system, that bridges some of the gaps between these two approaches. It has been successfully used in a number of GVHD biomarker discovery sets, and will serve as our discovery method in this proposal. (Paczesny et al. 2010; Ferrara et al. 2011; Wang et al. 2005)

The intact-protein analysis system, developed by Wang, depletes the most abundant plasma proteins and allows us to assay the concentrations of lower abundance plasma

proteins (Wang et al. 2005). When coupled with elements from Faca's work (Faca et al. 2006), the system becomes more robust and suited to purpose (Simpson and Greening 2011). Briefly, the protocol used in the seminal 2005 study relies on a tripartite separation. The samples should be pooled according to condition, then immuno-depleted of the most abundant plasma proteins using high performance liquid chromatography. Cysteine residues are then labeled with either light or heavy acrylamide isotopes according to the seminal work of Faca et al. (Faca et al. 2006). Then, proteins are separated by charge and hydrophobicity, then by molecular mass, followed by interrogation by mass spectrometric analysis (Wang et al. 2005). The separations are performed off line. Liquid-based isoelectric focusing separates the proteins by isoelectric point; proteins that have a similar isoelectric point co-elute in fractions according to the pH at which they carry no charge (Moritz and Simpson 2005). This procedure is followed by reverse phase high performance liquid chromatography, which relies on a hydrophobic stationary phase and a polar aqueous phase. Thus, hydrophilic proteins are eluted in the first fractions off the column, and hydrophobic fractions are eluted after the addition of a non-polar mobile phase such as acetonitrile (Wang et al. 2005). The final separation in this protocol is by mass, using sodium dodecyl sulfate – polyacrylamide gel electrophoresis. After separation by mass, protein expression is quantitatively visualized, by measuring the fluorescence of the cyanine dyes used to label the samples by condition earlier in the workflow (Tannu and Hemby 2006).

Proteins exhibiting differential expression between the two conditions are excised from the gels, washed, digested and analyzed via high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) (Wang et al. 2005). The spectra from each HPLC MS/MS run are analyzed for sequence matching. Previous studies have reported that some proteins can be inadequately separated by hydrophobicity and there may be some overlap in this reverse phase chromatography separation (Wang et al. 2005). After this has been taken into account, the identity of approximately 10-100 proteins in the micromolar to femtomolar, biologically relevant range that vary significantly in concentration between conditions will have been identified, with a false discovery rate of 5% (Paczesny 2013).

After execution of the discovery set and analysis of the mass spectrometry data, approximately 10 to 100 candidate spectra should have been identified that are selectively enriched in one condition or the other (Ferrara et al. 2011; Paczesny et al. 2010). These candidate spectra are mapped to a sequence database and protein identities are inferred through this mapping process. There is a now well-established false discovery rate of 5% in proteomic and microarray studies (Pawitan et al. 2005). A literature search, pathway analysis, the availability of immunoassays for validation studies, and in this case, expression in the tissue of interest using the human protein atlas (Berglund et al. 2008), help guide the selection of appropriate candidates for validation studies (Paczesny et al. 2010).

Once researchers have selected the candidate proteins, it is essential to ascertain that the candidate biomarkers of interest are expressed preferentially in the appropriate tissue and in the direction indicated by the discovery set. For example, if the candidate biomarker is up-regulated in the pathological condition, the biomarker should be preferentially expressed in tissue sections from patients exhibiting pathology as compared with matched specimens from disease-free control patients (Paczesny et al. 2010; Ferrara et al. 2011). Two small immunohistochemistry experiments should be run with appropriate controls. Tissue samples from age-matched patients who have not shown any signs of skin acute or chronic GVHD, but have exhibited documented drug reactions or skin manifestations of diseases other than acute or chronic GVHD should be compared with tissue samples from patients who have exhibited biopsy-proven skin chronic GVHD. The researcher will assay the tissue sections for the candidate biomarker and it should be present in the subcutaneous tissue, down to the dermis of affected patients. Tissue samples from cadaverous lungs from patients diagnosed with bronchiolitis obliterans and age-matched controls should be assayed for a candidate biomarker of lung chronic GVHD, and the biomarker should be preferentially expressed in this tissue when compared to the tissue from age-matched patients who have not shown any signs of bronchiolitis obliterans and have expired due to infection of the lung tissue with fungi or bacteria.

After the candidate biomarker has exhibited appropriate and selective up-regulation or down-regulation in the target tissues, validation sets must be established to determine whether or not a candidate biomarker has diagnostic and/or prognostic value.

In order to execute this next step of a biomarker identification study, a researcher will again rely on a strong institutional or multi-institutional banking study that can provide heparinized plasma samples from a large cohort of patients. Ideally these samples will have been collected fairly recently at regular intervals during the transplant period and at key events during the patient's post-transplant experience (Ferrara et al. 2011). Stability studies should be executed to ensure that the levels of the candidate biomarker are stable over the time period of the study; e.g., if plasma levels of the candidate biomarker are stable for two years, samples greater than two years old should not be included in the validation set (Parkitny et al. 2013). Furthermore, the general rule of thumb is that samples should not have been thawed and aliquoted for other studies more than twice. If samples cannot be obtained for a patient at several time points, they should not be included in analysis. For the purpose of a validation study, samples should be handled consistently across institutions and shipped to one institution to perform the biomarker assay itself.

Several studies have measured levels of biomarker at the onset of symptoms (Paczesny et al. 2009; Ferrara et al. 2011), as many hematopoietic stem cell transplant centers sample collection protocols require taking samples at or around the time of major changes in patient status. It might also be worthwhile to look for changes in the

biomarker in samples relatively close, and prior, to diagnosis, because a biomarker with a strong positive predictive value could be used in prospective studies to initiate treatment prior to symptoms developing (Paczesny et al. 2013).

In many validation studies, patients meeting some minimum criteria are all included for analysis. Generally these criteria include having available samples, and having had similar GVHD prophylaxis. These analyses are generally restricted to either an adult or pediatric patient population but do not have to be if it can be proven that introduction of this variable will not bias the data (Ferrara et al. 2011). Summary characteristics of all patients potentially included in the study should be determined prior to acquiring samples and assaying them for the candidate biomarkers of interest in order to show that the study is not a priori biased and is adequately powered to detect differences between outcomes.

For this particular study it will be exceedingly important to have many well-powered groups, which means it will likely require samples obtained from multiple hematopoietic stem cell transplant centers. These groups must be painstakingly parsed by chart review to determine their GVHD status i.e., what NIH criteria they met, e.g., acute, chronic, overlap, along with grading and staging (Baird et al. 2013), GVHD prophylaxis, GVHD treatment (i.e. systemic corticosteroid treatment), underlying disease, conditioning regimen, cell source and manipulation.

Ferrara required massive power; there were 1,1014 patients in the validation sets for the biomarker, regenerating islet –derived 3 alpha (Ferrara et al. 2011). A large sample population may be easily obtained for patients who have exhibited skin chronic

GVHD from the biobank of just one major hematopoietic stem cell transplant center. However, due to the relative paucity of patients whom develop bronchiolitis obliterans, the biobanks of many hematopoietic stem cell transplant facilities will have to be explored in order to get a least a ten-fold increase in sample population from the discovery set. The candidate biomarkers should be able to diagnose or predict the onset of disease (minimally) in both patients who exhibit tissue-specific chronic GVHD and, if the biomarker is a marker of effector cell response, not tissue-specific chronic graft-versus-host mediated damage, also in patients who exhibit other organ chronic GVHD. A candidate biomarker should be selective to the condition of interest. In the case of skin chronic GVHD, samples from control groups, including patients who developed drug rashes and other skin manifestations shown not to be chronic GVHD, patients who did not develop skin chronic GVHD, and patients who have chronic GVHD in other end organs, should have a significantly different level of expression than the target condition. Similarly, in the case of bronchiolitis, the biomarker should be able to discriminate between the target condition and control groups.

In addition to determining that the candidate biomarker can discriminate between the above-mentioned groups in the global set of patients, it will be worthwhile to statistically examine the effect of other criteria on candidate biomarker levels. It would not be surprising to see that previous GVHD (acute or chronic) treatment with corticosteroids affects biomarker levels, or that GVHD at different stages affects the levels of biomarkers (Berger et al. 2013). Additionally, the level of a biomarker at the

time of diagnosis might correspond to the NIH consensus criteria grade of skin or lung chronic GVHD at time of diagnosis.

Regardless of statistically significant differences between groups as determined using parametric or non-parametric means, the most important test of a biomarker is how it performs in non-parametric receiver-operator characteristic and area under the curve analysis. These methods, developed in the Second World War, yield optimal cut off points for biomarkers and help determine the negative and positive predictive values of the biomarker for the specific disease condition (Baker 2003). The curve plots the true positive rate (sensitivity) against the false positive rate ($1 - \text{specificity}$). The area under the curve summarizes the ability of the test to discriminate between the two conditions, in our case, the presence or absence of skin chronic GVHD. The area under the curve will range from a value of 1, indicating that the test perfectly predicts the presence or absence of the condition, to 0.5 indicating that the test has no ability to discriminate between the two conditions. In Figure 6, a set of receiver-operator characteristic curve from an acute GVHD are shown. In this study, a panel of biomarkers was analyzed to determine which biomarker(s) could diagnose acute lower gastro-intestinal GVHD in a series of patients who had post hematopoietic stem cell transplant diarrhea. The candidate biomarker displayed similar specificity and sensitivity and area under the curve to the four biomarker panel (Ferrara et al. 2011).

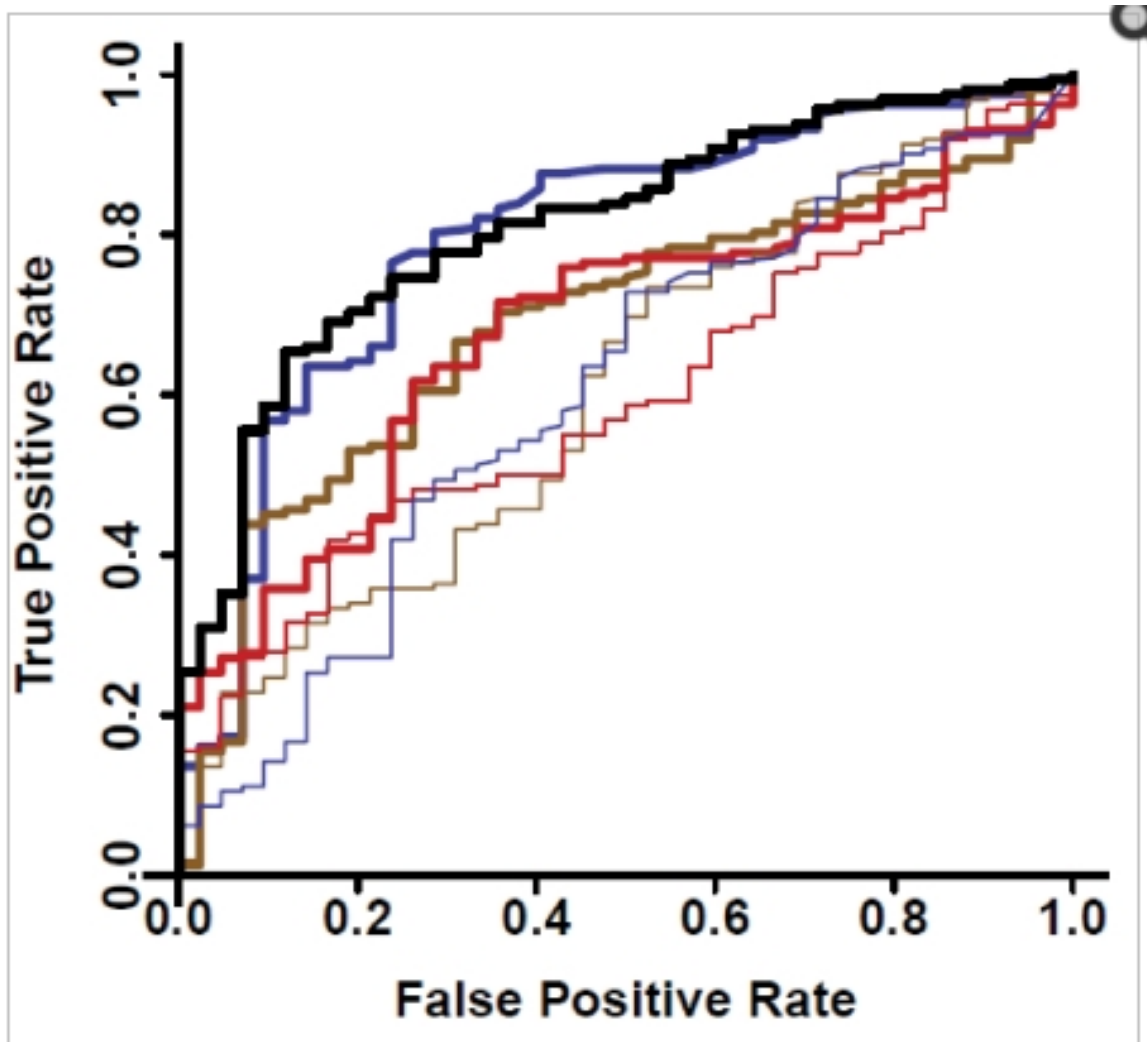


Figure 6: Receiver-Operator Characteristic curve for patients exhibiting post hematopoietic stem cell transplant diarrhea. Biomarkers were analyzed for 204 patients whom had diarrhea post hematopoietic stem cell transplant. The biomarker, regenerating islet-derived 3 alpha, is in thick dark blue, a panel of all biomarkers measured is in thick black. Other, inferior biomarkers are in different colors. The area under the curve for the biomarker regenerating islet-derived 3 alpha is 0.80, indicating that it is a useful biomarker. Adapted from Ferrara et al. 2011).

Ideally the biomarkers identified via the discovery set will have a sensitivity and specificity greater than that of the current gold standard of diagnosis, biopsy of the

affected tissue, i.e., the area under the curve for the identified biomarkers indicates correlation with a correct disease diagnosis with greater discrimination than biopsy.

The most important characteristic of a biomarker used for diagnosis is either a high positive predictive value or a high negative predictive value – of course, an ideal biomarker has both characteristics, but it is very unlikely that such a biomarker exists for an iatrogenic complex disease in the setting of another complex disease. A high positive predictive value indicates that when a biomarker indicates the disease or condition is present that it is highly accurate or “true”; that is to say the biomarker has a low false positive rate. A high negative predictive value indicates that when a biomarker indicates a disease is not present that it is also highly accurate or “true”, but in this case the biomarker has a low false negative rate. Generally a useful biomarker has one of these two characteristics (Hanash, Pitteri, and Faca 2008).

Biomarkers can also be used to stratify risk. Several studies have shown that a high or low (depending on the direction that indicates disease) biomarker level can stratify risk (Ferrara et al. 2011), much like the consensus NIH grading and staging of chronic GVHD (Baird et al. 2013) predicting non-relapse mortality over an appropriate time scale based on other criteria . This analysis could be easily conducted on a group of patients that have skin or lung chronic GVHD, using the statistical methods established by Fine (Fine and Gray 1999). Several studies have used acute GVHD biomarkers, alone or in a panel, to determine and predict treatment outcomes. Levine showed that a 6-protein biomarker panel, including the biomarkers tumor necrosis factor receptor-1,

Interleukin-2 receptor-alpha, Interleukin-8, hepatocyte growth factor, elafin, and regenerating islet-derived 3 alpha, were able to predict non-responders to treatment at 28 days post initiation of therapy and mortality at day 180 from onset (Levine et al. 2012).

A biomarker has greater clinical utility if it can predict response to treatment. Therefore appropriate non-parametric statistical analysis should be completed to determine whether high or low concentrations of biomarker within the affected group exhibit predictive value with respect to response to treatment, and predict the maximum clinical stage and grade of end-organ chronic graft-versus-host disease (MacMillan, DeFor, and Weisdorf 2010).

After the biomarker has been validated, and has exhibited a strong positive or negative predictive value, as well as an ability to stratify risk and predict response to treatment, follow-up prospective clinical trials must be initiated to truly prove the clinical utility of the biomarker.

We can utilize a model provided by Paczensy, shown in Figure 7 to evaluate the clinical utility of the biomarker in a phase two trial (Paczesny 2013). Briefly, in the intervention group, biomarker concentration can be used, either alone or in combination with established methods, to stratify the risk of non-relapse mortality in this subset of patients. Patients in the higher risk group can receive a best-in-class treatment prospectively, e.g. steroids plus a secondary agent, potentially extra corporeal photopheresis (Heshmati 2010), monoclonal antibody or other intervention (Choi and Reddy 2014). Patients in the control group receive the standard of care. The outcomes of

patients in both groups can be tracked, and hopefully the tailored interventions to biomarker levels can be used to decrease non-responsiveness to treatment and decrease morbidity and mortality in this vulnerable population of patients.

In the case of skin chronic GVHD, steroids plus a secondary agent as selected from a list of approved agents at a given hematopoietic stem cell transplant center, potentially extra corporeal photopheresis (Heshmati 2010), monoclonal antibody or other intervention (Choi and Reddy 2014). Patients in the control group receive the standard of care. The outcomes of patients in both groups can be tracked, and hopefully the tailored interventions to biomarker levels can be used to decrease non-responsiveness to treatment and decrease morbidity and mortality in this vulnerable population of patients. A similar protocol can be used in cases of lung chronic GVHD, wherein patients who exhibit abnormal pulmonary function tests, and abnormal findings on computerized tomography x-ray scans of the lungs at any point post-transplant, can be assayed for biomarker concentrations, and based on the concentration of the biomarker, can be assigned to either a two steroid, topical (inhaled) and systemic, or one steroid, topical (inhaled) regimen to treat their disease.

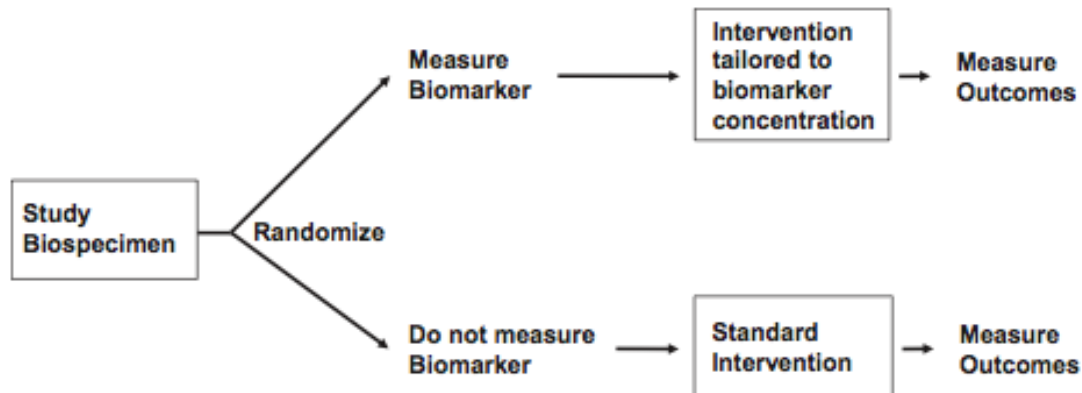


Figure 7: Fundamental design of a randomized trial to evaluate biomarker utility. Patients are randomized into two groups, those who receive the standard of care, and those for whom the validated biomarker is measured. Patients in the biomarker group receive an appropriate level of intervention, none, steroid, or best in class treatment (two agents) depending on their relative risk. The outcome variables are non relapse mortality, overall survival and the like.

This type of phase two clinical trial can be executed at a number of hematopoietic stem cell transplant centers. Furthermore, in the case of chronic lung GVHD, stratified biomarker concentration can be used to determine which patients will respond to either standard or enhanced treatment strategies and may be used to help fast-track some of these patients to acquire a lung transplant (Cheng et al. 2014). In addition to these clinical trials, the validated biomarkers may be used as part of a chronic GVHD biomarker panel to determine the relative risk of developing chronic GVHD in various allogeneic stem cell transplant settings, including the use of different sources of hematopoietic stem cell grafts (Ponce et al. 2014), including manipulated grafts, and in reduced intensity conditioning (Pollack et al. 2009), and haplo-identical hematopoietic

stem cell transplant, which is rapidly becoming an option at many first tier hematopoietic stem cell transplant facilities (Ballen et al. 2012).

CONCLUSION

In conclusion, biomarker discovery validation and detection may be accomplished leveraging proteomic techniques. These protein biomarkers speak directly to the pathogenesis of allo-reactivity in real time. Top-down and bottom-up mass spectrometry discovery techniques have identified many biomarkers for diverse conditions including both chronic (Devic et al. 2014) and acute GVHD (Paczesny et al. 2010) that would have most likely not been identified using hypothesis-driven research (Paczesny 2013). After validation under standard conditions, these biomarkers must be validated to determine their utility in diverse settings, including reduced intensity allogeneic hematopoietic stem cell transplant, and with various cell sources (Ponce et al. 2014). The goals of biomarker discovery in chronic GVHD is to be able to determine which patients will develop the condition, so as not to treat patients who have other skin conditions that may respond poorly to steroids or other treatment modalities increasing their risk of mortality, and to determine which patients will be non-responsive to the standard of care treatment, so that they may be treated more aggressively. Ultimately, the establishment of a repertoire of chronic GVHD biomarkers will help usher in an era of personalized medicine, and help determine the best intervention for each individual patient.

REFERENCES

- Addona, Terri A., Susan E. Abbatiello, Birgit Schilling, Steven J. Skates, D. R. Mani, David M. Bunk, Clifford H. Spiegelman, et al. 2009. "Multi-Site Assessment of the Precision and Reproducibility of Multiple Reaction Monitoring-Based Measurements of Proteins in Plasma." *Nature Biotechnology* 27 (7): 633–41. doi:10.1038/nbt.1546.
- Atarod, Sadaf, and Anne Mary Dickinson. 2013. "MicroRNAs: The Missing Link in the Biology of Graft-Versus-Host Disease?" *Frontiers in Immunology* 4: 420. doi:10.3389/fimmu.2013.00420.
- Baird, Kristin, Seth M. Steinberg, Lana Grkovic, Drazen Pulanic, Edward W. Cowen, Sandra A. Mitchell, Kirsten M. Williams, et al. 2013. "National Institutes of Health Chronic Graft-versus-Host Disease Staging in Severely Affected Patients: Organ and Global Scoring Correlate with Established Indicators of Disease Severity and Prognosis." *Biology of Blood and Marrow Transplantation* 19 (4): 632–39. doi:10.1016/j.bbmt.2013.01.013.
- Baker, Stuart G. 2003. "The Central Role of Receiver Operating Characteristic (ROC) Curves in Evaluating Tests for the Early Detection of Cancer." *Journal of the National Cancer Institute* 95 (7): 511–15. doi:10.1093/jnci/95.7.511.
- Bayraktar, Ulas D., Qaiser Bashir, Muzaffar Qazilbash, Richard E. Champlin, and Stefan O. Ciurea. 2013. "Fifty Years of Melphalan Use in Hematopoietic Stem Cell Transplantation." *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation* 19 (3): 344–56. doi:10.1016/j.bbmt.2012.08.011.
- Beretov, Julia, Valerie C. Wasinger, Peter Schwartz, Peter H. Graham, and Yong Li. 2014. "A Standardized and Reproducible Urine Preparation Protocol for Cancer Biomarkers Discovery." *Biomarkers in Cancer* 6: 21–27. doi:10.4137/BIC.S17991.
- Berger, M., E. Signorino, M. Muraro, P. Quarello, E. Biasin, F. Nesi, E. Vassallo, and F. Fagioli. 2013. "Monitoring of TNFR1, IL-2R α , HGF, CCL8, IL-8 and IL-12p70 Following HSCT and Their Role as GVHD Biomarkers in Paediatric Patients." *Bone Marrow Transplantation* 48 (9): 1230–36. doi:10.1038/bmt.2013.41.
- Berglund, Lisa, Erik Björling, Per Oksvold, Linn Fagerberg, Anna Asplund, Cristina Al-Khalili Szigyarto, Anja Persson, et al. 2008. "A Genecentric Human Protein Atlas for Expression Profiles Based on Antibodies." *Molecular & Cellular Proteomics* 7 (10): 2019–27. doi:10.1074/mcp.R800013-MCP200.
- Berz, David, Elise M McCormack, Eric S Winer, Gerald A Colvin, and Peter J Quesenberry. 2007. "Cryopreservation of Hematopoietic Stem Cells." *American Journal of Hematology* 82 (6): 463–72. doi:10.1002/ajh.20707.
- Bochennek, K., R. Esser, T. Lehrnbecher, W. Glienke, S. Wehner, S. Erben, J. Soerensen, et al. 2012. "Impact of Minimal Residual Disease Detection prior to Autologous Stem Cell

- Transplantation for Post-Transplant Outcome in High Risk Neuroblastoma.” *Klinische Pädiatrie* 224 (3): 139–42. doi:10.1055/s-0031-1301334.
- Champlin, Richard, Elias Jabbour, Partow Kebriaei, Paolo Anderlini, Borje Andersson, and Marcos de Lima. 2011. “Allogeneic Stem Cell Transplantation for Chronic Myeloid Leukemia Resistant to Tyrosine Kinase Inhibitors.” *Clinical Lymphoma, Myeloma & Leukemia* 11 Suppl 1 (June): S96–100. doi:10.1016/j.clml.2011.03.028.
- Chaturvedi, Anil K, Troy J Kemp, Ruth M Pfeiffer, Angelique Biancotto, Marcus Williams, Stella Munuo, Mark P Purdue, et al. 2011. “Evaluation of Multiplexed Cytokine and Inflammation Marker Measurements: A Methodologic Study.” *Cancer Epidemiology Biomarkers & Prevention* 20 (9): 1902–11. doi:10.1158/1055-9965.EPI-11-0221.
- Choi, Sung Won, and Pavan Reddy. 2014. “Current and Emerging Strategies for the Prevention of Graft-versus-Host Disease.” *Nature Reviews. Clinical Oncology* 11 (9): 536–47. doi:10.1038/nrclinonc.2014.102.
- Clift, R. A., C. D. Buckner, F. R. Appelbaum, S. I. Bearman, F. B. Petersen, L. D. Fisher, C. Anasetti, P. Beatty, W. I. Bensinger, and K. Doney. 1990. “Allogeneic Marrow Transplantation in Patients with Acute Myeloid Leukemia in First Remission: A Randomized Trial of Two Irradiation Regimens.” *Blood* 76 (9): 1867–71.
- Cohen, Edward P. 2015. “Cutaneous Manifestations of Graft-versus-Host Disease (GVHD).” Accessed February 9. http://www.uptodate.com/contents/cutaneous-manifestations-of-graft-versus-host-disease-gvhd?source=search_result&search=skin+chronic+graft+versus+host+disease&selectedTitle=1~3.
- Coron, Emmanuel, Valerie Laurent, Florent Malard, Marc Le Rhun, Patrice Chevallier, Thierry Guillaume, Jean-François Mosnier, Jean-Paul Galmiche, and Mohamad Mohty. 2014. “Early Detection of Acute Graft-versus-Host Disease by Wireless Capsule Endoscopy and Probe-Based Confocal Laser Endomicroscopy: Results of a Pilot Study.” *United European Gastroenterology Journal* 2 (3): 206–15. doi:10.1177/2050640614529283.
- “Definition of Biomarker - NCI Dictionary of Cancer Terms.” 2015. *National Cancer Institute*. Accessed February 3. <http://www.cancer.gov/dictionary>.
- Devic, Ivana, Min Shi, Mark M. Schubert, Michele Lloid, Kenneth T. Izutsu, Catherine Pan, Melody Missaghi, et al. 2014. “Proteomic Analysis of Saliva from Patients with Oral Chronic Graft-Versus-Host Disease.” *Biology of Blood and Marrow Transplantation* 20 (7): 1048–55. doi:10.1016/j.bbmt.2014.03.031.
- Faca, Vitor, Marc Coram, Doug Phanstiel, Veronika Glukhova, Qing Zhang, Matthew Fitzgibbon, Martin McIntosh, and Samir Hanash. 2006. “Quantitative Analysis of Acrylamide Labeled Serum Proteins by LC–MS/MS.” *Journal of Proteome Research* 5 (8): 2009–18. doi:10.1021/pr060102+.

- Fernand, J. P., P. Ravaut, S. Chevret, M. Divine, V. Leblond, C. Belanger, M. Macro, et al. 1998. "High-Dose Therapy and Autologous Peripheral Blood Stem Cell Transplantation in Multiple Myeloma: Up-Front or Rescue Treatment? Results of a Multicenter Sequential Randomized Clinical Trial." *Blood* 92 (9): 3131–36.
- Ferrara, James L. M., Andrew C. Harris, Joel K. Greenson, Thomas M. Braun, Ernst Holler, Takanori Teshima, John E. Levine, et al. 2011. "Regenerating Islet-Derived 3-Alpha Is a Biomarker of Gastrointestinal Graft-versus-Host Disease." *Blood* 118 (25): 6702–8. doi:10.1182/blood-2011-08-375006.
- Filipovich, Alexandra H., Daniel Weisdorf, Steven Pavletic, Gerard Socie, John R. Wingard, Stephanie J. Lee, Paul Martin, et al. 2005. "National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. Diagnosis and Staging Working Group Report." *Biology of Blood and Marrow Transplantation* 11 (12): 945–56. doi:10.1016/j.bbmt.2005.09.004.
- Fine, Jason P., and Robert J. Gray. 1999. "A Proportional Hazards Model for the Subdistribution of a Competing Risk." *Journal of the American Statistical Association* 94 (446): 496–509. doi:10.1080/01621459.1999.10474144.
- Goerner, Martin, Theodore Gooley, Mary E. D Flowers, Keith M Sullivan, Hans-Peter Kiem, Jean E Sanders, Paul J Martin, and Rainer Storb. 2002. "Morbidity and Mortality of Chronic GVHD after Hematopoietic Stem Cell Transplantation from HLA-Identical Siblings for Patients with Aplastic or Refractory Anemias." *Biology of Blood and Marrow Transplantation* 8 (1): 47–56. doi:10.1053/bbmt.2002.v8.pm11858190.
- Greinix, Hildegard T., Zoya Kuzmina, Roman Weigl, Ulrike Körmoczi, Arno Rottal, Daniel Wolff, Mateja Kralj, et al. 2014. "CD19+CD21low B Cells and CD4+CD45RA+CD31+ T Cells Correlate with First Diagnosis of Chronic Graft-versus-Host Disease." *Biology of Blood and Marrow Transplantation*. Accessed December 22. doi:10.1016/j.bbmt.2014.11.010.
- Hanash, Samir M., Sharon J. Pitteri, and Vitor M. Faca. 2008. "Mining the Plasma Proteome for Cancer Biomarkers." *Nature* 452 (7187): 571–79. doi:10.1038/nature06916.
- Han, Xuemei, Aaron Aslanian, and John R. Yates. 2008. "Mass Spectrometry for Proteomics." *Current Opinion in Chemical Biology* 12 (5): 483–90. doi:10.1016/j.cbpa.2008.07.024.
- Han, Xuemei, Mi Jin, Kathrin Breuker, and Fred W. McLafferty. 2006. "Extending Top-Down Mass Spectrometry to Proteins with Masses Greater Than 200 Kilodaltons." *Science* 314 (5796): 109–12. doi:10.1126/science.1128868.
- Heshmati, Farhad. 2010. "Extra Corporeal Photo Chemotherapy (ECP) in Acute and Chronic GVHD." *Transfusion and Apheresis Science: Official Journal of the World Apheresis Association: Official Journal of the European Society for Haemapheresis* 43 (2): 211–15. doi:10.1016/j.transci.2010.07.017.

- Jacobsohn, David A., Brenda F. Kurland, Joseph Pidala, Yoshihiro Inamoto, Xiaoyu Chai, Jeanne M. Palmer, Sally Arai, et al. 2012. "Correlation between NIH Composite Skin Score, Patient-Reported Skin Score, and Outcome: Results from the Chronic GVHD Consortium." *Blood* 120 (13): 2545–52; quiz 2774. doi:10.1182/blood-2012-04-424135.
- Keustermans, Genoveva C. E., Sanne B. E. Hoeks, Jenny M. Meerding, Berent J. Prakken, and Wilco de Jager. 2013. "Cytokine Assays: An Assessment of the Preparation and Treatment of Blood and Tissue Samples." *Methods, Cytokine Methods*, 61 (1): 10–17. doi:10.1016/j.ymeth.2013.04.005.
- Körbling, Martin, and Emil J. Freireich. 2011. "Twenty-Five Years of Peripheral Blood Stem Cell Transplantation." *Blood* 117 (24): 6411–16. doi:10.1182/blood-2010-12-322214.
- Krejci, Marta, Michael Doubek, Yvona Brychtova, Olga Stehlikova, Jana Chovancova, Boris Tichy, Hana Skuhrova Francova, et al. 2013. "Fludarabine with Cytarabine Followed by Reduced-Intensity Conditioning and Allogeneic Hematopoietic Stem Cell Transplantation in Patients with Poor-Risk Chronic Lymphocytic Leukemia." *Annals of Hematology* 92 (2): 249–54. doi:10.1007/s00277-012-1579-y.
- Kuzmina, Zoya, Hildegard T. Greinix, Roman Weigl, Ulrike Körmöczi, Arno Rottal, Sophie Frantal, Sandra Eder, and Winfried F. Pickl. 2011. "Significant Differences in B-Cell Subpopulations Characterize Patients with Chronic Graft-versus-Host Disease–associated Dysgammaglobulinemia." *Blood* 117 (7): 2265–74. doi:10.1182/blood-2010-07-295766.
- Lee, S.-E., B.-S. Cho, J.-H. Kim, J.-H. Yoon, S.-H. Shin, S.-A. Yahng, K.-S. Eom, et al. 2013. "Risk and Prognostic Factors for Acute GVHD Based on NIH Consensus Criteria." *Bone Marrow Transplantation* 48 (4): 587–92. doi:10.1038/bmt.2012.187.
- Levine, John E., Brent R. Logan, Juan Wu, Amin M. Alousi, Javier Bolaños-Meade, James L. M. Ferrara, Vincent T. Ho, Daniel J. Weisdorf, and Sophie Paczesny. 2012. "Acute Graft-versus-Host Disease Biomarkers Measured during Therapy Can Predict Treatment Outcomes: A Blood and Marrow Transplant Clinical Trials Network Study." *Blood* 119 (16): 3854–60. doi:10.1182/blood-2012-01-403063.
- MacMillan, Margaret L., Todd E. DeFor, and Daniel J. Weisdorf. 2010. "The Best Endpoint for Acute GVHD Treatment Trials." *Blood* 115 (26): 5412–17. doi:10.1182/blood-2009-12-258442.
- Marek, A., M. Stern, Y. Chalandon, M. Ansari, H. Ozsahin, T. Güngör, B. Gerber, et al. 2014. "The Impact of T-Cell Depletion Techniques on the Outcome after Haploidentical Hematopoietic SCT." *Bone Marrow Transplantation* 49 (1): 55–61. doi:10.1038/bmt.2013.132.
- McCarthy, Philip L., and Theresa Hahn. 2013. "Strategies for Induction, Autologous Hematopoietic Stem Cell Transplantation, Consolidation, and Maintenance for Transplantation-Eligible Multiple Myeloma Patients." *ASH Education Program Book* 2013 (1): 496–503. doi:10.1182/asheducation-2013.1.496.

- Mohty, Mohamad, and Anthony D. Ho. 2011. "In and out of the Niche: Perspectives in Mobilization of Hematopoietic Stem Cells." *Experimental Hematology* 39 (7): 723–29. doi:10.1016/j.exphem.2011.05.004.
- Moon, Joon Ho, Sang Kyun Sohn, Anna Lambie, Laura Ellis, Nada Hamad, Jieun Uhm, Vikas Gupta, et al. 2014. "Validation of National Institutes of Health Global Scoring System for Chronic Graft-Versus-Host Disease (GVHD) According to Overall and GVHD-Specific Survival." *Biology of Blood and Marrow Transplantation* 20 (4): 556–63. doi:10.1016/j.bbmt.2014.01.010.
- Morishima, Yasuo, Takehiko Sasazuki, Hidetoshi Inoko, Takeo Juji, Tatsuya Akaza, Ken Yamamoto, Yoshihide Ishikawa, et al. 2002. "The Clinical Significance of Human Leukocyte Antigen (HLA) Allele Compatibility in Patients Receiving a Marrow Transplant from Serologically HLA-A, HLA-B, and HLA-DR Matched Unrelated Donors." *Blood* 99 (11): 4200–4206.
- Moritz, Robert L., and Richard J. Simpson. 2005. "Liquid-Based Free-Flow Electrophoresis–reversed-Phase HPLC: A Proteomic Tool." *Nature Methods* 2 (11): 863–73. doi:10.1038/nmeth1105-863.
- Murphy, Elizabeth A., Stacy Stickney Ferguson, Nancy Atieno Omondi, Lisa C. Getzendaner, James L. Gajewski, Gary A. Goldstein, John R. Wingard, J. Douglas Rizzo, Navneet S. Majhail, and Navneet S. Mahjail. 2010. "The National Marrow Donor Program's Symposium on Patient Advocacy in Cellular Transplantation Therapy: Addressing Barriers to Hematopoietic Cell Transplantation." *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation* 16 (2): 147–56. doi:10.1016/j.bbmt.2009.08.004.
- Ofran, Yishay, and Jerome Ritz. 2008. "Targets of Tumor Immunity after Allogeneic Hematopoietic Stem Cell Transplantation." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 14 (16): 4997–99. doi:10.1158/1078-0432.CCR-08-0857.
- Paczesny, Sophie. 2013. "Discovery and Validation of Graft-versus-Host Disease Biomarkers." *Blood* 121 (4): 585–94. doi:10.1182/blood-2012-08-355990.
- Paczesny, Sophie, Thomas M. Braun, John E. Levine, Jason Hogan, Jeffrey Crawford, Bryan Coffing, Stephen Olsen, et al. 2010. "Elafin Is a Biomarker of Graft-versus-Host Disease of the Skin." *Science Translational Medicine* 2 (13): 13ra2. doi:10.1126/scitranslmed.3000406.
- Paczesny, Sophie, Oleg I. Krijanovski, Thomas M. Braun, Sung W. Choi, Shawn G. Clouthier, Rork Kuick, David E. Misek, et al. 2009. "A Biomarker Panel for Acute Graft-versus-Host Disease." *Blood* 113 (2): 273–78. doi:10.1182/blood-2008-07-167098.
- Paczesny, Sophie, Nisha Raiker, Sam Brooks, and Christy Mumaw. 2013. "Graft-versus-Host Disease Biomarkers: Omics and Personalized Medicine." *International Journal of Hematology* 98 (3): 275–92. doi:10.1007/s12185-013-1406-9.

- Parkitny, Luke, James H. McAuley, Patrick J. Kelly, Flavia Di Pietro, Barbara Cameron, and G. Lorimer Moseley. 2013. "Multiplex Cytokine Concentration Measurement: How Much Do the Medium and Handling Matter?" *Mediators of Inflammation* 2013: 1–13. doi:10.1155/2013/890706.
- Parks, Bryan A., Lihua Jiang, Paul M. Thomas, Craig D. Wenger, Michael J. Roth, Michael T. Boyne, Patricia V. Burke, Kurt E. Kwast, and Neil L. Kelleher. 2007. "Top-Down Proteomics on a Chromatographic Time Scale Using Linear Ion Trap Fourier Transform Hybrid Mass Spectrometers." *Analytical Chemistry* 79 (21): 7984–91. doi:10.1021/ac070553t.
- Pawitan, Yudi, Stefan Michiels, Serge Koscielny, Arief Gusnanto, and Alexander Ploner. 2005. "False Discovery Rate, Sensitivity and Sample Size for Microarray Studies." *Bioinformatics* 21 (13): 3017–24. doi:10.1093/bioinformatics/bti448.
- Pidala, Joseph, Jongphil Kim, Claudio Anasetti, Taiga Nishihori, Brian Betts, Teresa Field, and Janelle Perkins. 2011. "The Global Severity of Chronic Graft-versus-Host Disease, Determined by National Institutes of Health Consensus Criteria, Is Associated with Overall Survival and Non-Relapse Mortality." *Haematologica* 96 (11): 1678–84. doi:10.3324/haematol.2011.049841.
- Ponce, Doris M., Patrick Hilden, Christen Mumaw, Sean M. Devlin, Marissa Lubin, Sergio Giralt, Jenna D. Goldberg, et al. 2014. "High Day 28 ST2 Levels Predict for Acute Graft-versus-Host Disease and Transplant-Related Mortality after Cord Blood Transplantation." *Blood*, January, blood – 2014–06 – 584789. doi:10.1182/blood-2014-06-584789.
- Rozmus, Jacob, and Kirk R Schultz. 2011. "Biomarkers in Chronic Graft-versus-Host Disease." *Expert Review of Hematology* 4 (3): 329–42. doi:10.1586/ehm.11.27.
- Sarantopoulos, Stefanie, Kristen E Stevenson, Haesook T Kim, Nazmim S Bhuiya, Corey S Cutler, Robert J Soiffer, Joseph H Antin, and Jerome Ritz. 2007. "High Levels of B-Cell Activating Factor in Patients with Active Chronic Graft-versus-Host Disease." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 13 (20): 6107–14. doi:10.1158/1078-0432.CCR-07-1290.
- Schütt, P., J. Passon, P. Ebeling, A. Welt, S. Müller, K. Metz, T. Moritz, S. Seeber, and M. R. Nowrouzian. 2007. "Ifosfamide, Etoposide, Cytarabine, and Dexamethasone as Salvage Treatment Followed by High-Dose Cyclophosphamide, Melphalan, and Etoposide with Autologous Peripheral Blood Stem Cell Transplantation for Relapsed or Refractory Lymphomas." *European Journal of Haematology* 78 (2): 93–101. doi:10.1111/j.1600-0609.2006.00796.x.
- Servais, Sophie, Frédéric Baron, and Yves Beguin. 2011. "Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) after Reduced Intensity Conditioning." *Transfusion and Apheresis Science: Official Journal of the World Apheresis Association: Official Journal of the European Society for Haemapheresis* 44 (2): 205–10. doi:10.1016/j.transci.2011.01.019.

- Shimoni, Avichai, and Arnon Nagler. 2002. "Non-Myeloablative Hematopoietic Stem Cell Transplantation (NST) in the Treatment of Human Malignancies: From Animal Models to Clinical Practice." *Cancer Treatment and Research* 110: 113–36.
- Simpson, Richard J., and David W. Greening, eds. 2011. "Intact-Protein Analysis System for Discovery of Serum-Based Disease Biomarkers - Springer." In . *Methods in Molecular Biology* 728. Humana Press.
http://link.springer.com.ezproxy.bu.edu/protocol/10.1007/978-1-61779-068-3_4.
- Socié, Gérard, Judith Veum Stone, John R. Wingard, Daniel Weisdorf, P. Jean Henslee-Downey, Christopher Bredeson, Jean-Yves Cahn, et al. 1999. "Long-Term Survival and Late Deaths after Allogeneic Bone Marrow Transplantation." *New England Journal of Medicine* 341 (1): 14–21. doi:10.1056/NEJM199907013410103.
- Sorrer, Mohamed L., Michael B. Maris, Rainer Storb, Frederic Baron, Brenda M. Sandmaier, David G. Maloney, and Barry Storer. 2005. "Hematopoietic Cell Transplantation (HCT)-Specific Comorbidity Index: A New Tool for Risk Assessment before Allogeneic HCT." *Blood* 106 (8): 2912–19. doi:10.1182/blood-2005-05-2004.
- Steward, C. G., and A. Jarisch. 2005. "Haemopoietic Stem Cell Transplantation for Genetic Disorders." *Archives of Disease in Childhood* 90 (12): 1259–63.
doi:10.1136/adc.2005.074278.
- Storb, Rainer, Boglarka Gyurkocza, Barry E. Storer, Mohamed L. Sorror, Karl Blume, Dietger Niederwieser, Thomas R. Chauncey, et al. 2013. "Graft-versus-Host Disease and Graft-versus-Tumor Effects after Allogeneic Hematopoietic Cell Transplantation." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 31 (12): 1530–38. doi:10.1200/JCO.2012.45.0247.
- Tannu, Nilesh S., and Scott E. Hemby. 2006. "Two-Dimensional Fluorescence Difference Gel Electrophoresis for Comparative Proteomics Profiling." *Nature Protocols* 1 (4): 1732–42.
doi:10.1038/nprot.2006.256.
- Terwey, T. H., T. M. Le Duc, P. G. Hemmati, P. le Coutre, M. Nagy, P. Martus, B. Dörken, and R. Arnold. 2013. "NIH-Defined Graft-versus-Host Disease and Evidence for a Potent Graft-versus-Leukemia Effect in Patients with Acute Lymphoblastic Leukemia." *Annals of Oncology: Official Journal of the European Society for Medical Oncology / ESMO* 24 (5): 1363–70. doi:10.1093/annonc/mds615.
- Thomas, E. Donnall, Harry L. Lochte, Joe H. Cannon, Otto D. Sahler, and Joseph W. Ferrebee. 1959. "SUPRALETHAL WHOLE BODY IRRADIATION AND ISOLOGOUS MARROW TRANSPLANTATION IN MAN*." *Journal of Clinical Investigation* 38 (10 Pt 1-2): 1709–16.
- Ukena, Sya N., Robert Geffers, Stefanie Buchholz, Michael Stadler, and Anke Franzke. 2012. "Biomarkers for Acute and Chronic Graft-versus-Host Disease in Regulatory T Cells." *Transplant Immunology* 27 (4): 179–83. doi:10.1016/j.trim.2012.07.003.

- Vigorito, Afonso C., Paulo V. Campregher, Barry E. Storer, Paul A. Carpenter, Carina K. Moravec, Hans-Peter Kiem, Matthew L. Fero, et al. 2009. "Evaluation of NIH Consensus Criteria for Classification of Late Acute and Chronic GVHD." *Blood* 114 (3): 702–8. doi:10.1182/blood-2009-03-208983.
- Wang, Hong, Shawn G. Clouthier, Vladimir Galchev, David E. Misek, Ulrich Duffner, Chang-Ki Min, Rong Zhao, et al. 2005. "Intact-Protein-Based High-Resolution Three-Dimensional Quantitative Analysis System for Proteome Profiling of Biological Fluids." *Molecular & Cellular Proteomics: MCP* 4 (5): 618–25. doi:10.1074/mcp.M400126-MCP200.
- Wingard, J. R., S. Piantadosi, G. B. Vogelsang, E. R. Farmer, D. A. Jabs, L. S. Levin, W. E. Beschoner, R. A. Cahill, D. F. Miller, and D. Harrison. 1989. "Predictors of Death from Chronic Graft-versus-Host Disease after Bone Marrow Transplantation." *Blood* 74 (4): 1428–35.
- Xie, Lin-Na, Fang Zhou, Xi-Min Liu, Yuan Fang, Zhe Yu, Ning-Xia Song, and Fan-Sheng Kong. 2014. "Serum microRNA155 Is Increased in Patients with Acute Graft-versus-Host Disease." *Clinical Transplantation* 28 (3): 314–23. doi:10.1111/ctr.12314.

CURRICULUM VITAE

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Work Experience

Systems Business Analyst, Dana-Farber Cancer Institute, Cell Manipulation and Gene Transfer Core Facility (CMCF), Boston, MA.

March 2013 – current

- Create and revise standard operating procedures relevant to electronic systems.
- Script, Execute, Review test plans and validation plans in compliance with all applicable regulations and standards (software and hardware)
- Create detailed specifications for software purchase, stressing business needs and regulatory compliance
- Perform Solution Assessment and Validation for vendor's proposed software enhancements.

Cell Processing Technologist, Dana-Farber Cancer Institute, Cell Manipulation and Gene Transfer Core Facility (CMCF), Boston, MA.

September 2008 – March 2013

- Process cellular products for clinical use and clinical trials according to current good manufacturing practice (cGMP), current good tissue practices and FACT standards.
- Use flow cytometry to identify cell populations for clinical and research use.
- Create and revise standard operating procedures, and qualify equipment and procedures for use.
- Review processes and batch records for compliance with regulatory agencies and internal quality assurance.
- Create and execute validations in compliance with all applicable regulations and standards.

Medical Technician I, Massachusetts General Hospital, Boston, MA.

June 2005 – September 2008

- Worked in a high volume clinical chemistry laboratory, running various blood and urine tests, reporting values to clinicians, and processing samples.
- Maintained standards of precision and accuracy to ensure timely reporting of patient results.

Projects and publications:

Implementation of HPC screening test to determine the initiation of autologous apheresis

- The HPC screening test is currently in use at DFCI
- Data presented at AABB annual meeting, abstract published in supplement
- Full length article with expanded data set and analysis in publication

Validation and Implementation of the WBC-D test for determining the total nucleated cell count of HPC marrow products

- Currently in use by CMCF, used to determine recoveries for HPC marrow product manipulations

Memantine attenuates spatial and working memory impairment in Sprague Dawley rats induced by a zinc deficient diet

Advisor: Dr. Anne Jane Tierney

- Crafted a thesis, built a set of experiments and controls, ran the study, and analyzed and interpreted data in five months.

Characterization of cyclin-dependent kinase 5 (cdk5)/p35-regulated kinase (cprk): kinase activity and putative substrates

Advisors: Dr. Harish Pant, Dr. Sashi Kesavapany

- Worked independently on a project search for substrates of a novel kinase, culminating in a report on the work and a presentation and poster session at Building 10 on the NIH campus.

Regulation of neurofilament phosphorylation in the squid giant axon

Advisor: Dr. Phillip Grant

- Dissected and prepared squid giant axons
- Analyzed phosphorylation state of neurofilament proteins when exposed to various kinases using western blot and mass spectrometry

Education

Colgate University, Hamilton, New York

Bachelor of Arts Degree, May 2004

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Overall GPA: 3.34

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Skills

MS Office suite, Sunquest, EdgeCell, EPIC/Sunquest intergration, SAS (statistics)