Biomarkers for acute and chronic graft versus host disease: state of the art

Biomarkers for Acute and Chronic Graft vs. host Disease: State of the Art

Journal:	*Expert Review of Hematology*
Manuscript ID	EHM-2020--0152.R1
Manuscript Type:	Review (Invited)
Keywords:	circulating endothelial cells, cytokine, Extracellular vesicles, graft-versus-host disease, microbioma, microRNA, Natural Killer, proteomics, SNPs, regulatory T cells

Biomarkers for Acute and Chronic Graft vs. host Disease: State of the Art

Running title: Biomarkers in GVHD

Table: 1

Figures: 1

Word count: 8069

Abstract

Introduction: Despite significant advances in treatment and prevention, graft-versus-host disease (GVHD) still represents the main cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation. Thus, considerable research efforts have been made to find and validate reliable biomarkers for diagnosis, prognosis and risk stratification of GVHD.

Areas covered: In this review the most recent evidences on different types of biomarkers studied for GVHD, such as genetic, plasmatic, cellular markers and those associated with microbiome, were summarized. A comprehensive search of peer-review literature was performed in PubMed including meta-analysis, preclinical and clinical trials, using the terms: cellular and plasma biomarkers, graft-versus-host disease, cytokines, and allogeneic hematopoietic stem cell transplantation.

Expert opinion: In the near future, several validated biomarkers will be available to help clinicians in the diagnosis of GVHD, the identification of patients at high risk of GVHD development and in patients' stratification according to its severity. Then, immunosuppressive treatment could be tailored on each patient's real needs. However, more efforts are needed to achieve this goal. Although most of the proposed biomarkers currently lack validation with large scale clinical data, their study led to improved knowledge of the biological basis of GVHD, and ultimately to implementation of GHVD treatment.

Keywords: Circulating endothelial cells, Cytokines, Extracellular vesicles, Graft-versus-host disease, Microbiome, microRNA, Natural killer, Proteomics, Regulatory T-cells, SNPs.

Article highlights:

- Considerable research efforts have been done to find and validate relevant biomarkers for graft-versus-host disease (GVHD), as new tools to tailor the use of immunosuppressive drugs and to optimize GVHD management.
- The complex pathophysiology of GVHD makes the identification of reliable biomarkers challenging.
- A combined model including clinical and genetic variables could be able to correctly predict grades III-IV acute GVHD (aGVHD) and chronic GVHD (cGVHD).
- Changes in the composition of intestinal microbiota play a pivotal role in development of GVHD.
- T, B and Natural Killer (NK) cells are crucial in the maintenance of peripheral tolerance and impairment their function after allogeneic transplantation can lead to GVHD onset.
- aGVHD causes endothelial injury and circulating endothelial cells (CECs) are increased in affected patients, whether these cells can be used as valid biomarker is under evaluation.
- microRNAs (miRNAs) are small non-coding RNAs, mainly involved in the regulation of gene expression. In the context of allografting, many biomarker studies have been focused on the role of miRNAs involved in T-cell function in aGVHD.
- Extracellular vesicles (EVs) play an essential role in inter-cellular communications and their extraction from biological fluids requires relatively non-invasive protocols, which makes them attractive as biomarkers in GVHD setting.
- The development of high throughput technologies enabling the study of an entire spectrum of molecules led to the identification of a panel of cytokines which is, at the moment, the GVHD biomarker closer to clinical application.
- Despite many advances, the identification of valid GVHD biomarkers is still an unmet clinical need.

1. Introduction

Graft-versus-host disease (GVHD) can be a life-threatening complication of allogeneic hematopoietic stem cell transplantation (HSCT). Many advances have been made in GVHD treatment and prevention and several risk factors have been identified [1,2]. However, since morbidity and mortality related to both acute and chronic GVHD still represents a major concern, new diagnostic and therapeutic tools are needed to tailor the use of immunosuppressive drugs and to optimize GVHD prevention and treatment. With this purpose, considerable research efforts have been made to find and validate GVHD-relevant biomarkers.

However, the complex pathophysiology of GVHD that can be considered in a framework of distinct sequential phases of immune system dysregulation and cytokine production, makes the identification of reliable biomarkers challenging [3,4].

Potential applications of biomarkers in GVHD clinical trials and routine patient management include: (1) risk stratification for GVHD development; (2) diagnosis and assessment of GVHD severity, including distinguishing irreversible damage from continued disease activity especially in cGVHD; and (3) prediction of response to therapy [5].

Here, we summarize the main biomarkers being studied with the aim of helping clinicians in GVHD management, or, at least, of improving knowledge of GVHD. The correlation of each biomarkers with GVHD pathogenesis is illustrated in **Figure 1**, whereas the role of biomarkers (diagnostic, prognostic or predictive) in **Table 1**.

2. Pathogenesis of acute and chronic GVHD

GVHD biology is extremely complex and remains incompletely understood, involving intracellular signalling, soluble mediators, and cellular trafficking and interactions.

2.1. Donor and patient genetic background

In HSCT, although patients and donors can result HLA-identic according to major histocompatibility complex (MHC) antigens, they may differ for one or more proteins presented in form of HLA-peptide complexes to T cells acting as minor histocompatibility antigens (mHAs). Indeed, the human genome includes greater than 10^7 polymorphic sequences outside HLA and the role of mHAs is supported by genome-wide analysis of single-nucleotide polymorphisms (SNPs), which has revealed differences in the coding of amino acids and a variety of mechanisms related to DNA structural variation between recipients and donors [6-9]. Moreover, interesting results were obtained by genome-wide association studies (GWASs) [10], since risk of aGVHD is clinically increased in HSCT from unrelated as compared with related donors. Indeed, the percentage of

recipient coding SNPs mismatches was much larger for unrelated donor/recipient pairs than for sibling pairs [11]. Genome-wide arrays revealed that every 1% increase in genome-wide recipient mismatching is associated with a 20% increase in the risk of grades III-IV aGVHD [6]. Another GWAS study, including more than 3000 donor and recipient pairs, demonstrated a significant association between SNPs in the region of the MHC class II and the overall survival (OS) after HLA matched unrelated donor (MUD) HSCT [12]. Thus, HLA-mismatching in mHAs could likely explain most of the increased risk of GVHD after HSCT with unrelated donors [6].

Several studies showed a correlation between SNPs and genes involved in innate or adaptive immunity [e.g. interleukin(IL)-10, IL-6, IL-1 and its receptor, tumor necrosis factor-alfa (TNF-α), transforming growth factor-beta (TGF-β), cytotoxic T-lymphocyte antigen 4 (CTLA-4)] [13-15], although other studies failed to confirm this correlation [10,16,17]. More recently, a study performed on the large DISCOVeRY-BMT cohort showed that donor SNPs in the 2q12.1 region, which contains the IL-1 receptor ligand-1 (IL1R1) gene, were associated with elevated soluble suppression of tumorigenicity-2 (ST2) protein. Soluble ST2, which is the product of the IL1R1 gene, is a validated post-transplantation GVHD biomarker with a 4-fold risk of death for aGVHD, paving the way for potential use of this biomarker in donor selection process [18].

Despite the limitations of SNPs, Martinez-Laperche and colleagues were able to demonstrate a significant predictive value for their model which combined 25 SNPs on 12 cytokine genes of HLA matched related donors (MRD) and recipients with clinical variables (sex, age, female donor/male recipient, stem cell source, conditioning regimen and disease). In particular, the combined (clinical and genetic) model was able to correctly predict 100% of grades III-IV aGVHD cases (vs 88% of the model based on genetic variables only and 50% of that based on clinical variables only) and 80% of extensive cGVHD ones [19]. Using another combined model, Kim and colleagues were also able to predict the risk of aGVHD, but not of cGVHD [20].

2.2. Acute GVHD pathogenesis

In this complex genetic background, aGVHD pathophysiology can be simplified in a three-step model: (1) host antigen-presenting cells (APCs) activation due to tissue damage in the recipient by the conditioning regimen and antibiotic-mediated changes [21-23] in the microbiome (that cause a decline in protective microbial-derived metabolites); (2) subsequent donor T cells activation; and (3) pathogenic effector cells and inflammatory mediators producing the disease (**Figure 1**) [24].

Both in human and murine models, during the first step neutrophils, monocytes and inflammatory cells produce reactive oxygen species (ROS) as a consequence of tissue damage caused by chemo/radiotherapy and eventual infections, infiltrating the gastrointestinal (GI) tract [25-27]. The

endothelial cell injury, intimal arteritis and loss of microvessels (as observed in mice) [28,29], lead to the extracellular translocation of damage-associated molecular pattern (DAMPs) and pathogen-associated molecular patterns molecules (PAMPs). An additional consequence of GI tract damage is the perturbation of gut microbiota. Crypts in both the small and large intestine contain intestinal stem cells (ISCs) and Paneth cells. The latter act as guardians of the crypt in murine models [30], since their eosinophilic granules contain a wide range of antimicrobial peptides, including α-defensins, lysozyme, secretory phospholipase A2, and regenerating islet-derived protein 3α (REG3α). These are key elements of the intestinal mucosal barrier that protect from enteric pathogens and maintain intestinal homeostasis and microbiome stability through proliferation and maintenance of neighbouring ISCs [31,32]. Loss of commensal bacteria and microbial diversity during early post-transplantation period, often caused by mucositis and early use of systemic antibiotics, permits the overgrowth of pathogens associated with aGVHD [22,23,33,34]. In preclinical models, alteration of microbial metabolites such as short-chain fatty acids (SCFAs), tryptophan and butyrate, a histone deacetylase inhibitor, that modulates GVHD in an indoleamine-2,3-dioxygenase (IDO)-dependent manner, also has profound effects on mucosal immunity [35]. Thus, crypt damage, the break of integrity of the intestinal mucosa, and the loss of Paneth cells and their proteins result in dysbiosis. Furthermore, in a rodent model of GVHD has been observed that GVHD itself induces dysbiosis, thus fuelling a vicious pathogenetic circle [33].

All the mechanisms mentioned above lead finally to APCs activation. During the second phase, donor T cells are able to recognize allo-antigens on either host APCs (direct presentation) or donor APCs (indirect presentation). Over time during the post-transplant period, APCs change from primarily recipient origin to donor origin [36]. It is likely that direct presentation by host APCs is predominant during early stages of aGVHD, whereas indirect or cross-presentation by donor APCs is predominant in cGVHD.

During the last phase, the release of inflammatory cytokines by multiple cytotoxic effectors, such as phagocytes, NK cells, neutrophils and T cells, stimulates host tissues to produce inflammatory mediators directing effector cells into target organs through chemotaxis. This mechanism amplifies local tissue injury and leads to target tissue destruction, the final effect of humoral immunity in conjunction with direct cell-mediated cytolysis. A dysregulated uncontrolled cascade of immunological events and a lack of proper inhibitory regulatory systems represent the result of this complex biochemical process [4,37,38].

Finally, the interplay between cells and the extracellular matrix, together with the secretion of soluble factors, could be influenced by extracellular vesicles (EVs) trafficking in humans (see **section 3.3.2**) [39,40]. Indeed, biomolecules carried by EVs could be involved in many

physiological and pathological processes, being representative of their corresponding secreting cells.

2.3. Chronic GVHD pathogenesis

Similarly to aGVHD, also cGVHD development is associated with alteration in immune cell populations and immunoregulatory mediators [41].

The pathophysiology model of cGVHD, mostly derived from preclinical studies [42,43], can be divided into three phases: early inflammation caused by tissue injury (phase 1); thymic injury, dysregulated B-cell and T-cell immunity with auto- and/or allo-antibody production and consequent chronic inflammation (phase 2), culminating in tissue repair with fibrosis (phase 3) [3,44-46].

The pathogenesis of cGVHD begins with activation of host APCs expressed by damaged tissues and/or pathogens. As a consequence, donor T-cell proliferation and dysregulated inflammatory cytokine production [47,48] induce the activation of additional immune effector cells and perpetuate an adverse cycle of alloreactive inflammation.

Rodent models have been important to unravel immunological mechanisms of cGVHD. An important step in the phase 2 of cGVHD is the impairment in patient thymic function [49-53] due to thymic injury caused by aging, toxic effects of the conditioning regimen, prophylaxis with calcineurin inhibitors (CNIs), alloreactive T cells, and immunoglobulin deposition [54-56]. In rodent models, thymic dendritic cells and medullary and cortical thymic epithelial cells (mTECs and cTECs, respectively) are targeted by alloreactive T cells and pathologic antibodies, and their depletion leads to loss of central tolerance [43,57,58]. As a consequence of thymic injury, both positive and negative selection are affected by cGVHD [59]. Thus, potentially pathogenic T cells can escape from tolerization or deletion before peripheral export [60]. The net result is the proliferation of autoreactive and alloreactive CD4+ T cells producing IL-17α, which maintains inflammation, and the loss of regulatory-cell populations, including regulatory T cells (Tregs) [61], regulatory B cells (Bregs) [62,63], regulatory natural killer (NKreg) cells [64] and invariant natural killer T (iNKT) cells [65]. Lack of sufficient Tregs in the context of cGVHD can contribute to impaired peripheral tolerance, autoimmunity and further cGVHD development in preclinical models [66]. Besides, Tregs are capable to negatively regulate B-cell responses and selectively kill B cells [67], so their deficiency would predispose to a failure to control pathogenic B cells. As a matter of fact, several preclinical and clinical observations support the role of donor B cells in cGVHD development. The loss of B-cell tolerance, the altered B-cell homeostasis and the uncontrolled immunoglobulin production, possibly due to thymic dysfunction, could represent cGVHD triggering mechanisms [68-71]. Analysis in patients with cGVHD suggests that B cells

with a regulatory phenotype are both decreased and inactive [62,72]. Bregs can produce anti-inflammatory IL-10 and IL-35, being able to suppress the expansion of pathogenic CD4+ and CD8+ T cells through their immunoregulatory function, which may lessen the severity of sclerodermatous cGVHD in mice [73].

In phase 3, the coordination of T helper 2 cells (Th2) CD4+ cells, the up-regulation of TGF-β and IL-13, and the anti-PDGFR antibodies production, affect fibroblast collagen deposition, leading to aberrant tissue repair and fibrosis [73,74]. TGF-β-producing fibroblast activation by activated macrophages results in the production of extracellular matrix, which leads to tissue stiffness and sclerotic phenotype in murine models [45,74]. The production of isotype-switched immunoglobulin by differentiated B cells (plasma cells), fueled by B-cell activating factor (BAFF), results in pathogenic immunoglobulin deposition in various organs, which contributes to organ damage and fibrosis.

3. Biomarkers role

Biomarkers to predict the risk of both aGVHD and cGVHD before and after transplantation might represent a turning point in the therapeutic approach of HSCT patients. As a consequence, in the past two decades a growing number of preclinical and clinical studies evaluated target molecules that looked promising in this field [5,75].

3.1. Microbiome

The human GI tract is inhabited by a multitude of microorganisms, referred to as the intestinal microbiota, while their associated genomes are defined as the microbiome. Among an estimated 10^{14} individual bacteria, most are non-pathogen anaerobic commensal bacteria: bacterial phyla Firmicutes and Bacteroidetes are prevalent in the intestinal microbiota, followed by Proteobacteria, Fusobacteria and Actinobacteria. Microbiota shares a lot of variability between individuals, with only one third of bacterial species being common between two individuals [76-78]. In the last years, new molecular techniques have allowed a better knowledge of the human microbiota composition, including 16S rRNA sequencing and the unbiased high-resolution method of metagenomics shotgun sequencing, while *in situ* hybridization and PCR are used to identify and quantify bacteria [77].

Studies focusing on the human GI microbiota composition before and after HSCT reported a drastic loss of bacterial diversity after transplantation, often accompanied by the expansion of a single taxon (mainly Enterococci), and loss of Clostridia species known to produce SCFAs: these changes are linked to an increased risk of infections and GVHD, and to decreased OS [77,79-81]. Indeed, death from GVHD in HSCT has been associated with low bacterial species diversity [79], and the lack of Blautia Luti in the stool microbiota [82] **(Table 1)**.

Golob and colleagues prospectively collected stool samples in patient from pre-transplantation until day 100 post-transplantation: a total of 694 stool profiles plus 36 microbiotas from healthy donors were analyzed, showing an association between impaired bacterial species diversity and severe aGVHD. In particular, some organisms, like oral Actinobacteria and oral Firmicutes, appeared to be predictive of severe aGVHD. On the contrary, patients that did not develop GVHD had microbiota similar to those observed in healthy donors, with dominance of Bacterioidaceae and/or Lachnospiraceae [83]. A subsequent study published in 2018 confirmed these observations, showing that patients with aGVHD had an impaired microbiota diversity at the time of engraftment, with dominance by a single microbiota family (i.e. Gammaproteobacteria and Enterobacteriaceae) and a loss of Lachnospiraceae and Ruminococcaceae which influences Tregs/Th17 balance with the reduction of Tregs [84].

A predictive model based on human gut microbiome sequencing has been recently proposed [85]. Stool and samples of 150 evaluable patients from two centers were collected at preconditioning, transplantation and neutrophil engraftment. The algorithm, defined as gut microbiota score (GMS), defined distinct risks of developing severe aGVHD based on selected features of intestinal bacteria. GMS has been shown to correlate with Tregs/Th17 balance and the amount of proinflammatory cytokines.

Changes in microbiome structure cause a change in intestinal metabolites, which may play a role in aGVHD severity, and could be used as surrogate markers for microbiome characterization as suggested by both murine and human studies [35,86-89].

Besides, it has been observed that urinary 3-indoxyl sulfate (3-IS, a major conjugate of indole) levels at the time of HSCT and early thereafter were associated with gut microbiota disruption. In patients, low levels of 3-IS predicted higher transplant-related mortality (TRM), with intestinal GVHD as the primary cause [90]. Indeed, 3-IS could contribute to GVHD protection by stimulating Th2 responses and monitoring of urinary 3-IS levels may be a feasible approach to monitor microbiome changing.

In 2020 Payen and colleagues combined the study of intestinal bacteria and their metabolites at GVHD onset. A weekly stool sample was collected at the time of aGVHD onset in 35 patients, whereas 35 non-GVHD patients were used as controls. Bacterial count and diversity were significantly lower at GVHD onset in patient with severe aGVHD; patients with mild aGVHD had microbiota similar to controls. As previously demonstrated, Lachnospiraceae (e.g. Blautia) and Ruminococcaceae were significantly reduced in patients with severe aGVHD. Besides, this study suggests that butyrate may be a potential marker of GVHD and that propionate and acetate may be associated with disease severity [91].

Finally, a recent paper highlighted the relationship between microbiota and cGVHD, analyzing stool and blood samples from 54 cGVHD patients around day 100 post HSCT and 171 controls: plasma concentrations of butyrate and propionate were significantly lower in cGVHD patients, reflecting a different microbiota composition in stool samples. Furthermore, abundance of Akkermansia and Streptococcus were found to positively correlate with cGVHD, while abundance of Clostridium and Lactoclostridia seemed to be protective. These data showed that the lasting microbiome damage may impact on cGVHD. SCFA administration might gain a therapeutic role in this setting [92].

Unfortunately, specific microbiota alterations relevant for GVHD development were not always consistent among studies. Although the microbiome is an exciting and rapidly emerging area, several important challenges had to be faced by researchers. Each patient has a peculiar microbiome, reinforcing the notion that there is no single "healthy" microbiome profile. Each host has a unique biological relationship with its microbiota, characterized by complex molecular interactions within specific niches in the gut. Differences in the microbiome exist across age, cultures and geography. Moreover, faecal bacterial community can be detected by different procedures, sampling and storage protocols, as well as DNA extraction methods. In addition, animal experiments depend on several factors such as genetic background, sterility of the environment and diet, so researchers should consider these challenges carefully when designing experiments. Strategic collaboration of clinicians, microbiologists, molecular biologists, computational scientists, and bioinformaticians could represent the ideal paradigm for success in this field in the near future.

3.2. Cellular biomarkers

As detailed *above*, immune cells play a key role in the pathogenesis and in the control of graft-versus-host interaction and several of them have been identified as potential biomarkers of aGVHD and cGVHD, with a predominant role of T lymphocytes **(Table 1)**.

3.2.1. T and NK cells

Peripheral tolerance after allogeneic HSCT significantly contributes to establishment of a balance between recipient tissues and donor-derived immunity. Tregs are crucial in the maintenance of this process. A significant reduction of Tregs has been observed in aGVHD but also in cGVHD and this decrease was correlated with severity of manifestations [93]. Thus, Tregs relative counts could be a prognostic biomarker for GVHD [93]. In addition, the frequencies of Tregs at onset of aGVHD could predict the response to GVHD treatment in patients [94]. Tregs were shown to be reduced also in patients with cGVHD compared to healthy subjects, regardless of a previous diagnosis of

aGVHD [95], as demonstrated by reduced frequency of CD4+CD25+Foxp3+ T lymphocytes [93,96,97]. Furthermore, a striking inverse correlation between the percentages of Tregs and CD8+ cytolytic T cells in patients with cGVHD emerged [95]. In a paediatric cohort, Tregs have been specifically identified as associated with freedom from cGVHD. Fewer data are available on aGVHD. In both adult and paediatric cohorts, a higher CD4+/CD8+ T-cell ratio was reported in patients who develop aGVHD [98-100].

CD31 is an excellent marker of recent thymic emigrants, within Foxp3+ Tregs population in humans [96]. Higher percentages of CD4+CD45RA+CD31+ T cells have been seen on day 100 post-HSCT and at onset of cGVHD, and they significantly could predict later development of cGVHD [101], showing both prognostic and diagnostic role [102].

Raised levels of Th17 lymphocytes strongly correlate with the inflammatory process taking place in aGVHD and active cGVHD, as demonstrated by Dander et al. [103]. Interestingly, an inverse relationship between Tregs and Th17 has been shown, not only in peripheral blood but also in sites of active cGVHD in patients [103,104]. Within conventional T and Tregs, a CD4+CD146+CCR5+ subpopulation with a Th17 profile has been described, which increased in patients with cGVHD [105]. Moreover, the expansion of this subset appeared to be an early event in the pathogenesis of GI GVHD and might assume prognostic value in predicting development of aGVHD in subjects underwent allogeneic HSCT [106].

Another subset of T helper, follicular helper T cells (cTFH), were reduced in patients with active cGVHD and their phenotype is skewed toward Th2/Th17 subsets, capable of inducing B-cell activation and immunoglobulin production. A linear relationship between active cTFH and clinical grading of cGVHD was shown [107].

CD30 expression appeared to be increased on effector and central memory CD8+ T cells in patients with aGVHD [108], acting as diagnostic biomarker and, possibly, as a therapeutic target.

In addition to T cells, also NK cells were correlated with GVHD. In this regard, a delayed reconstitution of the immune-regulatory $CD56^{bright}$ NK cells was observed in patients with aGVHD and cGVHD [109]. An inverse relationship between $CD56^{bright}$ NK-cell levels and aGVHD onset was shown, thus revealing a role as early prognostic biomarker [109]. NK cells could be also predictors for cGVHD [109]: lower proportion of $CD56^{bright}$ NK regulatory cells results in higher rate of cGVHD and it is associated with higher levels of C-X-C motif chemokine ligand 10 (CXCL10), a chemokine secreted in response to IFN-gamma (IFN-γ) that binds to C-X-C receptor 3 (CXCR3) and is involved in T-cell recruitment to inflamed tissue [64].

3.2.2. B cells

The cytokine BAFF plays a critical role in normal B-cell maturation and survival. In the context of B-cell lymphopenia after HSCT, high soluble BAFF levels promote the selection and expansion of autoreactive B cells [69,70]. Indeed, BAFF levels and B-cell counts are significantly higher in patients with active cGVHD than in those without [110]. BAFF/B-cell ratio is an important indicator of cGVHD [110-112] and it is related to the cGVHD grading [113]. Elevated ratios were observed in patients with hypogammaglobulinemia and related to onset and activity of cGVHD [114]. Increased values were observed in patients with lung involvement, confirming the validity of a potential biomarker for early diagnosis of bronchiolitis obliterans syndrome (BOS), also in asymptomatic patients [115]. Conversely, low BAFF/B-cell ratios after umbilical cord blood transplantation have also been associated with a low incidence of cGVHD [111].

Within the first year after HSCT, early severe B-cell lymphopenia is followed by the progressive normalization of B-cell count. In the context of GVHD, elevated immature/transitional CD21- B-cell and low CD27+ memory B-cell counts have been seen in patients with active cGVHD [112] and are associated with more frequent infectious complications [116]. Increased absolute count of CD19+CD21^{low} B cells was observed at the onset of *de novo* cGVHD [117]. Furthermore, the same panel, assessed at day 100 after HSCT, was predictive for subsequent development of quiescent and progressive cGVHD [101,112]. Association between low CD19+CD21^{low} levels and activity and severity of cGVHD has been revealed also in a paediatric cohort [118]. The resolution of cGVHD correlated with the normalization of CD19+CD21^{low} levels, thus CD19+CD21^{low} might help with distinction between active vs inactive cGVHD [118]. Similar results were observed in patients responding to extracorporeal photopheresis (ECP) [119]. Along with high BAFF/B-cell ratios, elevated levels of CD19+CD21^{low} lymphocytes were observed in patients with new onset of pulmonary cGVHD and long-lasting BOS, hinting a possible role as biomarker for early diagnosis of this serious GVHD manifestation [115]. Memory B-cells are profoundly reduced in patients developing cGVHD [114,116,120]. Active cGVHD has been related to a low proportion of CD19+CD27+ memory B-cells and persistent low memory B-cell counts predicted an increased risk of cGVHD during later follow-up in a paediatric cohort [118]. Unlike cGVHD, late-onset aGVHD was associated with higher levels of unswitched memory B cells and transitional B cells in children [121].

3.2.3 Invariant NKT

Invariant natural killer T cells (iNKT) are a rare subset of lymphocytes that co-express T-cell and NK-cell markers selectively activated by glycolipid antigens presented by CD1d and characterized by an invariant TCR α-chain named Vα24jα18 in humans [122]. iNKT are further distinguished in

two different subsets, based on CD4 expression, characterized by a different cytokine profile with CD4$^-$iNKT secreting higher amounts of IFN-γ than IL-4, resulting in a Th1 bias [123]. Both preclinical mouse models and clinical observations have shown that iNKT cells are capable to modulate immune response and may represent an important marker to predict the occurrence of aGVHD.

In a seminal preclinical work by Lan et al. [124], in which mice received reduced intensity conditioning (RIC), total lymphoid irradiation and anti-thymocyte globulin (ATG), recipient iNKT cells preferentially survived because of radioresistance resulting in aGVHD abrogation. Such effect was dependent on host T cells IL-4 secretion [125,126] and on donor T cells STAT-6 expression [127]. iNKT lead to donor Th2 polarization and resulted in donor Tregs expansion [65,126,128]. Donor Tregs were not dispensable since the protective effect of α-galactosylceramide infusion was lost when donor Tregs cells were depleted [65,129].

Consistently, both iNKT recovery after transplantation and graft iNKT dose were found to correlate with the occurrence of aGVHD in humans. In one of the earliest study involving 106 patients undergoing HSCT either from a MRD or MUD after a myeloablative conditioning (MAC), the number of iNKT were significantly reduced in patients developing aGVHD after a bone marrow graft [130]. In another study comprising 71 subjects undergoing MRD or MUD transplantation either after MAC or RIC [131], the iNKT/T-cell ratio, analyzed between day 15 and day 90 after transplantation, was found to represent a reasonable surrogate marker of iNKT reconstitution. Patients with $\geq 1x10^{-3}$ ratio had lower chance to develop aGVHD and Cytomegalovirus infection, resulting in lower incidence of NRM and enhanced OS. Day 15 iNKT/T-cell ratio could efficiently discriminate the risk of aGVHD with an AUC of 0.812 and may represent a reliable marker to identify patients at higher risk to develop aGVHD [131]. In another report comprising 78 patients receiving peripheral blood stem cell (PBSC) MRD transplantation [132], a higher graft content of iNKT was associated with a lower chance of aGVHD: 31% vs 64% for iNKT $\geq$ vs $<0.057x10^6$/Kg. This effect was particularly evident for CD4$^-$iNKT cells and may be due to its direct cytotoxic activity against CD1d-expressing mature myeloid dendritic cells [123]. Malard et al. [133] analyzed a cohort of 80 patients receiving MRD, MUD or mismatched unrelated (MMUD) transplantation employing RIC and ATG, and found that a higher iNKT cell graft content ($>0.11x10^6$/Kg) was associated with improved GVHD-free and progression-free survival (GRFS). This effect was mainly due to a reduced incidence of disease relapse and cGVHD. In another report [134], only pre-transplantation donor CD4$^-$iNKT expansion capacity was associated with aGVHD in patients receiving a PBSC graft. Of note, donor iNKT graft content did not correlate with donor age, while iNKT recovery was lower with increasing recipient age. Therefore, even if we are unable to select a

particular donor to improve iNKT reconstitution, iNKT graft content and post-transplantation recovery represent important makers to identify patients at higher risk of aGVHD.

3.2.4. Circulating endothelial cells

The endothelium was recently recognized as a significant target of donor T-cell alloreactivity, being involved in the pathogenesis of aGVHD, especially when steroid refractoriness is established [135]. Preclinical mouse models and clinical observations showed that markers of neovascularization and endothelial damage are associated with the occurrence of aGVHD and may be useful to predict its onset and response to front-line therapy. In a seminal work, Penack et al. [28] described that a hallmark of target organs of aGVHD is represented by neovascularization driven by donor-derived vasculogenesis in a murine model. Donor circulating endothelial progenitor cells (EPCs) were found to be increased in the peripheral blood of mice with aGVHD, resulting in increased vascularization of the liver, the colon and the bone marrow. These observations are consistent with histologic findings in the human counterpart, where donor bone marrow derived vasculogenesis was found to contribute to neovascularization of the skin and the intestine of patients with aGVHD [136,137]. Given this background, the authors proposed a model linking endothelial cells (ECs) and aGVHD [138]: in the early phase, endothelial damage is caused by different toxic agents such as the conditioning regimen (chemo- or radio-therapy), infections or drugs (such as CNIs); in the second phase, vessels react by recruiting new donor-derived ECs and neovascularization takes place; in the third phase, alloreactive T cells target the endothelium and blood vessels are destroyed.

Two main implications stem from these findings: 1) inhibition of vasculogenesis may ameliorate aGVHD; 2) markers of endothelial damage and circulating endothelial cells (CECs) may be helpful in the diagnosis of aGVHD in humans. To address the first question Penack et al. [28] treated mice with an anti-VE cadherin antibody named EG410, that specifically bind and depletes EPCs, resulting into abrogation of aGVHD and increased survival. The second question has been answered by several clinical reports investigating whether markers of ECs injury or CECs are increased in patients with aGVHD. Almici et al. [139] described a significant relative increase in the number of CECs in patients with aGVHD relative to patients without aGVHD (44% vs 0%, p=0.04). An inverse correlation was found at the time of the engraftment, with a reduced number of CECs in patients who will develop aGVHD compared to aGVHD free subjects. Of relevance, not the absolute numbers, but the relative changes (either incremental or decremental) of CECs were significantly associated with aGVHD and engraftment. Moreover, CECs values were a marker of response to aGVHD therapy because they returned to pre-transplantation levels in responding patients. In a subsequent report, Almici et al. [140] confirmed these observations and described that

CECs changes after allogeneic HSCT are a dynamic phenomenon influenced by conditioning regimen, engraftment, infections and immunosuppressive treatments. Nevertheless, enumeration of CECs is still not a standardized procedure yet, since the CellSearch system (CED identified as $CD146^{+}CD106^{+}CD45^{-}$cells) or polychromatic flow-cytometry (CEC defined as $CD34^{+}CD45^{-}CD146^{+}$cells) bring complimentary, but not completely overlapping, results [141].

3.3. ~~6.~~ Plasma biomarkers

In addition to altered immune cells subsets count, the balance between pro- and anti-inflammatory cytokines, chemokines, soluble cell receptors and proteins, miRNAs, EVs, and immune activated biomarkers plays a key role in both the initiation of GVHD and its progression. Serum biomarkers associated with GVHD, reflecting underlying biological process of both aGVHD and cGVHD, have shown not only to be useful in predicting GVHD occurrence before the onset of clinical symptoms, but also to estimate its risk and to predict patient's outcomes **(Table 1)**.

3.3.1. miRNAs

MicroRNAs (miRNAs) are small non-coding RNAs, mainly involved in the regulation of gene expression, thus controlling crucial cellular processes, including cell proliferation, differentiation, apoptosis [142,143]. Easily detectable in body fluids, their measurement represents a potential non-invasive diagnostic and predictive tools for many diseases [144], including GVHD upon HSCT [143,145,146].

In the context of HSCT, most studies on miRNAs focused on their role in T-cell function and aGVHD onset, while less data are available on miRNAs role as biomarkers of cGVHD.

The increased expression of miR181a, regulating T-cell maturation and TCR signalling, was able to prevent aGVHD onset in rodent models of HSCT [147,148]. Similarly, the expression of miR146a, a negative regulator of inflammation prevalently expressed in Tregs, has been shown to have a protective role against aGVHD. In agreement, low expression of miR146a was associated with an increased incidence of aGVHD during the first 28 days post-HSCT [149] and mice treated with a mimic of miR146a showed a reduced aGVHD severity and a better prognosis [150]. On the contrary, miR155, physiologically involved in B and T-cell proliferation and in controlling effector and regulatory T-cell function [151], was upregulated in T cells from mice developing aGVHD after allogeneic HSCT. Moreover, miR155 expression blockade ameliorated aGVHD severity and survival in mice [152].

The clinical relevance of miR181a and miR155 has been confirmed in patients receiving allogeneic HSCT. MiR155 level was increased and miR181a expression was reduced before aGVHD onset and their levels directly and inversely correlated with aGVHD severity, respectively [148,153]. Together these data suggest that miRNAs could act in concert to regulate inflammatory responses, thus indicating that the investigation of miRNA clusters as aGVHD biomarkers could be more informative than the study of a single miRNA.

In this context, the upregulation of miR20a and 15a and the downregulation of miR181a, miR146a, miR30b-5p, and miR374-5p showed diagnostic utility for aGVHD, being differentially expressed already 14 days post-HSCT in patients who later developed aGVHD [154].

Moreover, a global microRNA expression profiling on skin biopsies identified the miR34a-3p and miR503-5p as related to cutaneous aGVHD. The expression of these two miRNAs, together with miR34a-5p appeared to be elevated also in the sera of aGVHD patients [155].

Investigating a specific plasma miRNA signature on 196 patients underwent HSCT, Xiao and coworkers identified a 4-miRNA-based diagnostic panel, composed by miR423, miR199a-3p, miR93 and miR377, which was able to early predict the occurrence and severity of aGVHD [156]. This evidence was further confirmed by the observation that increased levels in serum and urine of miR423, miR199, and miR93 at day 14 after HSCT could predict the occurrence of aGVHD [157,158].

Furthermore, circulating miR26b, miR374a, miR28-5p, miR489 and miR671-3p could improve early diagnosis of aGVHD [159], similarly to what was observed for miR194 and miR518f in a cohort of 24 lymphoma patients [160].

3.3.2. Extracellular vesicles

In recent years, the rapidly growing research area on EVs has demonstrated they have essential role in inter-cellular communications, thus being involved in many physiological and pathological juxtacrine signalling processes (i.e. immune response modulation, inflammation, cancer, cardiometabolic, neurologic and infectious diseases) [161]. EVs are membrane enclosed organelles circulating in biological fluids, and are secreted by virtually all cell types carrying different biomolecules, including nucleic acids (DNA [162,163], RNA [164,165] and miRNAs), proteins [166-169], lipids, and carbohydrates [40,170,171].

EVs extraction from biological fluids requires relatively non-invasive protocols, which makes them attractive as biomarkers. Moreover, the biomolecules carried by EVs could be representative of the secreting cells, representing an attractive tool for molecular diagnosis, together with molecules presented on the EVs surface. Thus, the analysis of their molecular cargo is emerging as a new form

of "liquid biopsy", useful to gain insights about disease clinical features, biological characteristics, and therapy response, without being invasive.

Wu et al. observed that EVs from endothelial origin were altered after HSCT before aGVHD onset [172], while Lia et al. investigated the potential role of EVs as biomarkers of GVHD [173]. In this latter study, a statistically significant correlation between three EVs membrane antigens (CD146, CD31, CD140a) with the risk of developing aGVHD was retrospectively observed. Furthermore, all the three biomarkers showed a significant level change on EVs membrane before the onset of aGVHD [173]. Correlation of EVs membrane antigen (CD146 and CD31) with aGVHD onset was also confirmed by preliminary results in a new prospective study [174].

In the last years, exploratory study on miRNA profiles has been extended also on EVs. As a matter of fact, EVs are also natural carriers of miRNAs and they support the release of such molecules to recipient cells, protecting them from degradation of plasma ribonucleases. MiR155 is an example of miRNA which is dysregulated and upregulated in aGVHD patients in both cell free- and EVs carried form. Furthermore, a study *in vitro* demonstrated that after TNF-α stimulation of human umbilical vein ECs, EVs are enriched in miR155 [175]. Levels of miR155 were significantly higher in EVs compared to plasma level in aGVHD patients as well as in mouse models. Moreover, inhibition of miR155 by loading antagomir-155 inside EVs reduced differentiation toward Th1, Th9 and Th17 cells and skewed differentiation towards Th2 cells and Tregs, which ameliorated clinical and pathological manifestations of aGVHD. In another preliminary study, expression change of miR155, with miR100 and miR194b before aGVHD onset was also observed in serum EVs [174]. Circulating miR423, miR199, and miR93 in serum derived EVs could be also used as diagnostic and prognostic biomarkers for aGVHD [158].

Further studies are needed to better characterize and define EVs as reliable biomarkers for aGVHD, and no data are presently available in cGVHD context. Nevertheless, the aforementioned findings strongly suggest the potential clinical applications of EVs in this setting.

3.3.3. Cytokines and chemokines

Cytokines and chemokines are small proteins which are secreted by various cells to mediate immune response and trafficking, to recruit immune cells to inflammation sites and to promote T-cell differentiation and expansion. These effects are mediated by their binding to specific receptors on target cells which modify transcription patterns, protein expression, and migratory behaviour [176,177]. Moving from the evidence that a "cytokine storm" is a peculiar feature of aGVHD, cytokines and their receptors have been explored as potential target for studies on biomarkers on patients [19] **(Table 1)**, among others, IL-2, IL-6, IL-12, IL-15, IL-18, IL-33, IFN-γ and TNF-α

[4,178,179]. Soluble TNF-α is an inflammatory mediator of tissue damage during aGVHD and its role in the pathogenesis of aGVHD prompted the evaluation of TNF-blocking agents for the treatment of steroid-refractory aGVHD (SR-aGVHD) [180-182]. Moreover, an increase in the concentration of serum TNF-α and tumor necrosis factor receptor 1 (TNFR1) at day 7 post-HSCT were associated with disease severity and survival in both adult and paediatric patients [183,184]. Nevertheless, this association is not specific enough to allow TNF-α to be used as an independent predictor for GVHD development. Indeed, an increase of TNF-α was also observed, in both human and murine models, before major transplant-related complications such as interstitial pneumonitis and veno-occlusive disease [183,185].

IL-2 is a cytokine primarily produced by CD4+ T cells after their activation, being implicated in T-cell activation and proliferation. Monoclonal antibodies (mAbs) directed towards IL-2 receptor α-chain (IL-2Rα), such as daclizumab or basiliximab, are currently used to inhibit activated alloreactive T cells in patients with SR-aGVHD and GI aGVHD [186,187]. Furthermore, soluble IL-2Rα levels were increased prior to clinical onset of aGVHD in many studies and could be used to predict both aGVHD development and severity [188]. Nevertheless, sIL-2Rα levels, like TNF-α ones, rise also in the setting of other transplant-related complications [189]. In addition, sIL-2Rα levels can be altered by CNIs, commonly used for GVHD prophylaxis [190].

IL-33 is a member of the IL-1 superfamily of cytokines, thought to be released from damaged tissues as an alarmin to induce Th2 responses and repair through ST2 receptor. Dysregulation of ST2/IL-33 signalling pathway was originally described in the context of different inflammatory diseases[191]. Several preclinical and clinical studies investigated the contribution of CCR5 and its ligands in the development of GVHD [192]. In preclinical models, CCR5+CD8+ T lymphocytes significantly contributed to liver GVHD. Administration of anti-CCR5 antibody dramatically reduced the infiltration of donor T cells into the liver, and consequently reduced hepatic damage [193]. The Seattle group reported that lymphocyte infiltrated in the skin samples of patients with aGVHD were predominantly CCR5+ T cells [194]. Genetic polymorphisms of cytokines and chemokines correlated with GVHD risk and severity in patients [195]. Studies showed that genetic deletion of CCR5 in both human recipients and donors resulted in a decreased incidence of GVHD [196,197]. Recently, a phase 2 study showed the safety and efficacy of CCR5 antagonist maraviroc for the prophylaxis of GVHD in patients undergoing HSCT [198,199].

Several preclinical and clinical studies investigated the contribution of CCL8, CXCL10, and CXCL11 with its ligands, in the development of aGVHD [192,200,201]. Soluble BAFF (sBAFF), CXCL-9, CXCL-10, CXCL-11, ST2 and IL-33 have been frequently associated with the risk of cGVHD in several studies [64,110,202-204]. In addition to its correlation with aGVHD [205], ST2

possess a good cGVHD predictive ability in combination with CXCL9, matrix metalloproteinase 3 (MMP3), and osteopontin (OPN). Furthermore, this 4-biomarker panel showed a significant correlation with cGVHD diagnosis and severity, together with NRM [203]. The receptor for CXCL9, CXCL10 and CXCL11 is CXCR3, predominantly expressed on the surface of Th1 cells. Recent studies demonstrated the involvement of CXCR3 ligands in GVHD pathogenesis, revealing a central role for chemokine-mediated recruitment of CXCR3+ T cells in this setting [204]. The hypothesis that CXCR3 ligands (in particular CXCL9) act as gatekeepers for tissue distribution of alloreactive T cells in cGVHD was supported by high levels of these chemokines in oral, ocular, and mucosal cGVHD [206,207]. Furthermore, CXCR3 ligands could be associated with progression, organ dysfunction and complications of cGVHD. However, the importance of these chemokines in the diagnosis of cGVHD needs to be further evaluated.

Most studies showed an increase in pro-inflammatory cytokines in cGVHD cases, including TNF-α, IL-6, IL-17, IL-1β, IL-8, sIL-2Rα shed by activated T cells and IL-1Rα [103,206-208]. Conversely, only TGF-β, IL-15, IL-4 and IL-2 were decreased at cGVHD onset [209,210]. Patients with lower serum levels of IL-15 at day 7 post-HSCT had 3-fold higher risk of developing cGVHD subsequently [209], and IL-15 levels were inversely correlated with CD8+ T cells levels, cellular subtypes involved in the development of cGVHD. Severity of established cGVHD correlated with level of TNF-α, IL-6, and IL-1β [41]. Among all the cGVHD biomarkers, a decreased level of sIL-2R and sBAFF were associated to response to therapy [208,211],whereas increased levels were associated with higher mortality [211].

Since infectious diseases, immune factors, immunosuppressive drugs and aGVHD can modify the levels of the aforementioned biomarkers, their predictive value remains difficult to establish. Indeed, only CXCL9 was confirmed as a robust cGVHD biomarker in a recent multicenter study [203]. Moreover, the levels of some biomarkers (e.g. BAFF and CXCL9) could be modified also by corticosteroids [110,202]. Hence, many efforts are needed to independently validate the role of these promising biomarker candidates in large studies.

3.3.4. Proteomics

The development of high throughput technologies enabling the study of an entire spectrum of molecules has provided new insights into the comprehension of the pathophysiological mechanism of a disease and the identification of novel biomarkers useful in diagnosis and prognostic stratification. In the context of GVHD, both mass spectrometry (MS)-based and non-MS-based approaches have been used to identify candidate biomarkers [212].

Among the non-MS-based assays, antibody microarrays have been used to screen aGVHD biomarkers in peripheral blood. By investigating 120 proteins on plasma of HSCT patients, Paczesny and coworkers identified 8 potential biomarkers for aGVHD diagnosis. After their validation by enzyme-linked immunosorbent assay (ELISA), the authors defined a 4-protein composite biomarker panel [IL-2Rα, TNFR1, IL-8, and hepatocyte growth factor (HGF)] able to discriminate patients with and without aGVHD and to predict their survival independently from GVHD severity [213]. Subsequently, the same group identified three organ-specific biomarkers, namely the skin-specific marker elafin, the GI GVHD-specific biomarker REG3α and cytokeratin-18 fragments (KRT18), which correlated with intestinal and liver GVHD, with prognostic significance [214-216]. In particular, REG3α, a marker secreted by Paneth cells associated with GI epithelial injury and repair, was validated as predictive and prognostic biomarker of aGVHD and showed higher diagnostic precision for lower GI GVHD. [214]. Furthermore, REG3α concentrations at GVHD onset predicted response to therapy at 4 weeks, NRM and survival [217]. All above-mentioned biomarkers are unfortunately not specific for liver GVHD, being produced also in the setting of other transplant-related [214].

By combining this knowledge, a multicenter, randomized, 4-arm phase 2 clinical trial (Clinical Trials Identifier NCT00224874) was undertaken to investigate whether the above-mentioned 6 markers (IL-2Rα, TNFR1, IL-8, HGF, elafin and REG3α) could be able to define the prognosis and therapy response of aGVHD patients. The authors demonstrated that the 6-protein biomarker measurement at GVHD onset, 2 and 4 weeks after treatment start was able to identify therapy non-responsive patients and to predict their survival [218].

Two ST2 isoforms having opposite roles have been described: a transmembrane form and a soluble isoform, that acts as a decoy receptor sequestering IL-33. During aGVHD, an altered secretion of soluble ST2 by intestinal cells was observed in experimental models [191]. Soluble ST2 measurement at the time of GVHD diagnosis was validated as a biomarker for treatment-resistant aGVHD, and elevated circulating ST2 at day 7 or 14 post-HSCT could also be predictive of NRM following HSCT [219,220].The combined measurement at day 7 post-HSCT of TNFR1, IL-2Rα, REG3α and ST2 enabled the development of a predictive algorithm (Mount Sinai Acute GVHD International Consortium or MAGIC), mainly based on ST2 and REG3α concentrations after one week of systemic glucocorticoid treatment, to early identify patients at high risk for lethal GVHD and NRM in a multicenter cohort of 1287 patients [221]. In agreement, the prognostic relevance of the measurement of REG3α and ST2 was recently confirmed in a cohort of 110 consecutive patients who underwent haploidentical HSCT. In this report, higher plasma levels of REG3α and ST2 were associated with a higher incidence of grade II-IV aGVHD and NRM, but only 30 day after

transplantation [222]. MAGIC algorithm demonstrated to be accurate when measured at multiple time-points during the course of transplantation, implying that it could be a used as a response biomarker to provide a dynamic tool that predicts outcomes more accurately than change in clinical symptoms [223].

In addition to the biomarker panels described above, other biomarker combinations, including ST2+REG3α+TNFR1 [224], ST2+TNFR1, TIM3+TNFR1+IL6 [225], ST2+TIM3 [226], have been investigated in the plasma of HSCT patients to predict the aGVHD occurrence and severity.

Since different patient cohorts and different endpoints have been considered to test each biomarker combination, it is difficult to define the best one to identify robust early indicator(s) of GVHD occurrence and severity. In this regard, Etra and coworkers tested the ability of the different biomarker combination to predict 1-year lethal GVHD on more than 500 patients. Their results demonstrated that the measurement of ST2 and REG3α serum levels had a higher predictive accuracy [227].

In addition to circulating aGVHD biomarkers, a wide range of MS-based proteomic approaches have been recently used on urine and saliva. In this regard, by using capillary electrophoresis and tandem mass spectrometry, Wessinger and colleagues identified in urine a 17-peptide panel, named aGVHD_MS17, able to accurately and early detect aGVHD patients and to predict grade III-IV aGVHD [228]. In addition, the same group defined a second 14-peptide biomarker for early diagnosis of cGVHD [229]. Similarly, Chiusolo and coworkers trough high-performance liquid chromatography combined with electrospray-ionization mass spectrometry identified two proteins, S100A8 and S100A9, as possible aGVHD biomarkers [230].

4. Conclusions

In the past years, advances in technology have permitted the discovery of numerous biomarkers for diagnosis, prognosis and prediction of GVHD together with progress in understanding its pathophysiology. Importantly, studies on biomarkers improved our understanding of GVHD pathogenesis and found new pathways that could be targeted by antibodies or small molecules, finally contributing to the development of new effective treatments for GVHD. For instance, given the important role of IL-6 in GVHD pathogenesis [231], a trial assessing tocilizumab for the treatment of cGVHD therapy is ongoing (NCT02174263) [46]. Also ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitors, which is critical for B-cell survival, proliferation, and migration [232], is an irreversible inhibitor of IL-2 inducible kinase [233] and interfere with many cytokine cascades involved in GVHD development [3,44,45], has been recently introduced in SR-GVHD treatment

Although many specific and sensitive biomarkers for both aGVHD and cGVHD have been identified over the past decades, much efforts are still needed to move from bench to daily clinical practice.

Expert opinion

Reliable and validated biomarkers in GVHD have many potential future applications. First, implementation of donor and patient selection for HSCT, thanks to genetic polymorphisms or microbiome modifications studies that might identify patterns at high risk of GVHD development. Furthermore, the identification of specific changes in microbiome, cellular subtypes and/or panel of molecules specific for GVHD could greatly help physicians in GVHD management and in differential diagnosis between GVHD and other post-HSCT complications which sometimes can be challenging. Similarly, biomarkers that allow an early recognition of patients who are very likely to develop SR-GVHD could led to early treatment intensification in those patients, and a treatment sparing in the others.

Weak points are the limited sample size of patient cohorts and the lack of large-scale validation. Furthermore, more efforts should be done to minimize confounding variables, such as different conditions, other than GVHD, affecting the same biomarker. Another important limit to their widespread use is the complexity and the cost of the analyses necessary to measure biomarkers. Finally, to be employed in the clinical setting, biomarkers should be detectable on easy-to-collect samples with non-invasive methods, however most of the reported studies were in line with that.

In the future, a special interest should be placed on the role of microbiome in GVHD pathogenesis, although its role is not so easy to establish due to the frequent controversial results. The concept that the manipulation of GI microorganisms (i.e. through different use of antibiotics, immunomodulators, chemotherapy) could eventually influence the development of aGVHD, and likely cGVHD and other HSCT complications as well, is fascinating. Other promising and growing sections are EVs, miRNAs and CECs, which play a crucial role in cellular interactions. We are not completely aware of all the potential information that these markers carry, but more research in these fields will hopefully led to greater knowledge in pathophysiology and eventually to the possibility of interfering with cellular crosstalk.

Given the complexity of mechanisms involved, it is likely that a panel of markers rather than a single one will result meaningful. Furthermore, biomarkers for aGVHD will be available to clinicians in the next future, as the research is more advanced in this setting. Hopefully, validated

markers for cGVHD will follow, as the interest and the number of published studies is growing over the time also in this field.

Among the illustrated biomarkers, the plasmatic panel proposed by MAGIC consortium is the most advanced in clinical development. The first trial which include a panel of biomarkers (TNFR1, ST2, and REG3α) [224] to assign GVHD treatment has been conducted by the Bone Marrow Transplant Clinical Trials Network (Clinical Trials Identifier NCT02806947), and the results should be available in the near future.

At present, the search of GVHD biomarkers is not part of clinical routine, and their application remains restricted to clinical trials. Nevertheless, biomarkers studies play an important role in improving the knowledge of the complex pathophysiology of aGVHD and cGVHD. Finally, a better understanding of the mechanisms leading to GVHD has been crucial to the introduction on new treatments for SR-GVHD.

References

1. Holler E, Greinix H, Zeiser R. Acute Graft-Versus-Host Disease. In: Carreras E, Dufour C, Mohty M, et al., editors. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham (CH): Springer Copyright 2019, EBMT and the Author(s). 2019. p. 323-30.
2. Wolff D, Lawitschka A. Chronic Graft-Versus-Host Disease. In: Carreras E, Dufour C, Mohty M, et al., editors. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham (CH): Springer Copyright 2019, EBMT and the Author(s). 2019. p. 331-45.
3. Zeiser R, Blazar BR. Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. N Engl J Med. 2017 Dec 28;377(26):2565-2579.
4. Zeiser R, Blazar BR. Acute Graft-versus-Host Disease - Biologic Process, Prevention, and Therapy. N Engl J Med. 2017 Nov 30;377(22):2167-2179.
   **** This review provides an overview of acute GVHD biology.**
5. Paczesny S, Hakim FT, Pidala J, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: III. The 2014 Biomarker Working Group Report. Biol Blood Marrow Transplant. 2015 May;21(5):780-92.
6. Martin PJ, Levine DM, Storer BE, et al. Genome-wide minor histocompatibility matching as related to the risk of graft-versus-host disease. Blood. 2017 Feb 9;129(6):791-798.
7. Santos N, Rodriguez-Romanos R, Nieto JB, et al. UGT2B17 minor histocompatibility mismatch and clinical outcome after HLA-identical sibling donor stem cell transplantation. Bone Marrow Transplant. 2016 Jan;51(1):79-82.
8. Mullally A, Ritz J. Beyond HLA: the significance of genomic variation for allogeneic hematopoietic stem cell transplantation. Blood. 2007 Feb 15;109(4):1355-62.
9. Warren EH, Zhang XC, Li S, et al. Effect of MHC and non-MHC donor/recipient genetic disparity on the outcome of allogeneic HCT. Blood. 2012 Oct 4;120(14):2796-806.
10. Adom D, Rowan C, Adeniyan T, et al. Biomarkers for Allogeneic HCT Outcomes. Front Immunol. 2020;11:673.
11. Martin PJ. Increased disparity for minor histocompatibility antigens as a potential cause of increased GVHD risk in marrow transplantation from unrelated donors compared with related donors. Bone Marrow Transplant. 1991 Sep;8(3):217-23.
12. Sucheston-Campbell L, Preus L, Spellman S, et al. Functional Single Nucleotide Polymorphisms (SNPs) in the Major Histocompatibility Complex (MHC) Class II Region Are Associated with Overall Survival (OS) after HLA Matched Unrelated Donor BMT: Results from the Discovery-BMT Study. Biology of Blood and Marrow Transplantation. 2016;22(3):S72-S73.
13. Dickinson AM. Non-HLA genetics and predicting outcome in HSCT. Int J Immunogenet. 2008 Aug;35(4-5):375-80.
14. Elmaagacli AH, Koldehoff M, Landt O, et al. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. Bone Marrow Transplant. 2008 May;41(9):821-6.
15. Kim DH, Jung HD, Lee NY, et al. Single nucleotide polymorphism of CC chemokine ligand 5 promoter gene in recipients may predict the risk of chronic graft-versus-host disease and its severity after allogeneic transplantation. Transplantation. 2007 Oct 15;84(7):917-25.
16. Karaesmen E, Rizvi AA, Preus LM, et al. Replication and validation of genetic polymorphisms associated with survival after allogeneic blood or marrow transplant. Blood. 2017;130(13):1585-1596.
17. Martin PJ, Fan W, Storer BE, et al. Replication of associations between genetic polymorphisms and chronic graft-versus-host disease. Blood. 2016 Nov 17;128(20):2450-2456.

18. Karaesmen E, Hahn T, Dile AJ, et al. Multiple functional variants in the IL1RL1 region are pretransplant markers for risk of GVHD and infection deaths. Blood Adv. 2019 Aug 27;3(16):2512-2524.
19. Martinez-Laperche C, Buces E, Aguilera-Morillo MC, et al. A novel predictive approach for GVHD after allogeneic SCT based on clinical variables and cytokine gene polymorphisms. Blood Adv. 2018 Jul 24;2(14):1719-1737.
20. Kim DD, Yun J, Won HH, et al. Multiple single-nucleotide polymorphism-based risk model for clinical outcomes after allogeneic stem-cell transplantation, especially for acute graft-versus-host disease. Transplantation. 2012 Dec 27;94(12):1250-7.
21. Peled JU, Devlin SM, Staffas A, et al. Intestinal Microbiota and Relapse After Hematopoietic-Cell Transplantation. J Clin Oncol. 2017 May 20;35(15):1650-1659.
22. Shono Y, Docampo MD, Peled JU, et al. Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. Sci Transl Med. 2016 May 18;8(339):339ra71.
23. Weber D, Jenq RR, Peled JU, et al. Microbiota Disruption Induced by Early Use of Broad-Spectrum Antibiotics Is an Independent Risk Factor of Outcome after Allogeneic Stem Cell Transplantation. Biology of Blood and Marrow Transplantation. 2017;23(5):845-852.
24. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet. 2009 May 2;373(9674):1550-61.
25. Reinhardt K, Foell D, Vogl T, et al. Monocyte-induced development of Th17 cells and the release of S100 proteins are involved in the pathogenesis of graft-versus-host disease. J Immunol. 2014 Oct 1;193(7):3355-65.
26. Schwab L, Goroncy L, Palaniyandi S, et al. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. Nature medicine. 2014 Jun;20(6):648-54.
27. Socie G, Mary JY, Lemann M, et al. Prognostic value of apoptotic cells and infiltrating neutrophils in graft-versus-host disease of the gastrointestinal tract in humans: TNF and Fas expression. Blood. 2004 Jan 1;103(1):50-7.
28. Penack O, Henke E, Suh D, et al. Inhibition of neovascularization to simultaneously ameliorate graft-vs-host disease and decrease tumor growth. Journal of the National Cancer Institute. 2010;102(12):894-908.
29. Riesner K, Shi Y, Jacobi A, et al. Initiation of acute graft-versus-host disease by angiogenesis. Blood. 2017 Apr 6;129(14):2021-2032.
30. Sasaki N, Sachs N, Wiebrands K, et al. Reg4+ deep crypt secretory cells function as epithelial niche for Lgr5+ stem cells in colon. Proc Natl Acad Sci U S A. 2016 Sep 13;113(37):E5399-407.
31. Clevers HC, Bevins CL. Paneth cells: maestros of the small intestinal crypts. Annu Rev Physiol. 2013;75:289-311.
32. Sato T, van Es JH, Snippert HJ, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature. 2011 Jan 20;469(7330):415-8.
33. Eriguchi Y, Takashima S, Oka H, et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of alpha-defensins. Blood. 2012 Jul 5;120(1):223-31.
34. Taur Y, Jenq RR, Perales MA, et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. Blood. 2014 Aug 14;124(7):1174-82.
35. Mathewson ND, Jenq R, Mathew AV, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. Nat Immunol. 2016 May;17(5):505-513.
36. Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. Blood. 2007 Jul 1;110(1):9-17.

37. Beilhack A, Schulz S, Baker J, et al. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood. 2005 Aug 1;106(3):1113-22.
38. Zeiser R. Biology-driven developments in the therapy of acute graft-versus-host disease. Hematology. 2018;2018(1):236-241.
39. Lia G, Di Vito C, Cerrano M, et al. Extracellular Vesicles After Allogeneic Hematopoietic Cell Transplantation: Emerging Role in Post-Transplant Complications. Frontiers in immunology. 2020;11:422-422.
*** This is a detailed review on Extracellular Vesicles and their application in HSCT.**
40. Shah R, Patel T, Freedman JE. Circulating Extracellular Vesicles in Human Disease. N Engl J Med. 2018 Nov 29;379(22):2180-2181.
41. Barak V, Schaffer FL, Nisman B, et al. Cytokine dysregulation in chronic graft versus host disease. Leukemia & lymphoma. 1995;17(1-2):169-173.
42. Srinivasan M, Flynn R, Price A, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. Blood. 2012 Feb 9;119(6):1570-80.
43. Wu T, Young JS, Johnston H, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. J Immunol. 2013 Jul 1;191(1):488-99.
44. Cooke KR, Luznik L, Sarantopoulos S, et al. The Biology of Chronic Graft-versus-Host Disease: A Task Force Report from the National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease. Biology of Blood and Marrow Transplantation. 2017;23(2):211-234.
45. MacDonald KP, Blazar BR, Hill GR. Cytokine mediators of chronic graft-versus-host disease. J Clin Invest. 2017 Jun 30;127(7):2452-2463.
46. MacDonald KP, Hill GR, Blazar BR. Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. Blood. 2017 Jan 5;129(1):13-21.
**** This review provides an overview of chronic GVHD biology.**
47. Socie G. Disease severity in chronic graft-versus-host disease: doctors' gut feeling versus biostatistics? Haematologica. 2014 Oct;99(10):1534-6.
48. Socie G, Ritz J. Current issues in chronic graft-versus-host disease. Blood. 2014 Jul 17;124(3):374-84.
49. Weinberg K, Blazar BR, Wagner JE, et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. Blood. 2001 Mar 1;97(5):1458-66.
50. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nat Rev Immunol. 2012 May 11;12(6):443-58.
51. Clave E, Busson M, Douay C, et al. Acute graft-versus-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. Blood. 2009 Jun 18;113(25):6477-84.
52. Fallen PR, McGreavey L, Madrigal JA, et al. Factors affecting reconstitution of the T cell compartment in allogeneic haematopoietic cell transplant recipients. Bone Marrow Transplant. 2003 Nov;32(10):1001-14.
53. Gray DH, Seach N, Ueno T, et al. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood. 2006 Dec 1;108(12):3777-85.
54. Fletcher AL, Lowen TE, Sakkal S, et al. Ablation and regeneration of tolerance-inducing medullary thymic epithelial cells after cyclosporine, cyclophosphamide, and dexamethasone treatment. J Immunol. 2009 Jul 15;183(2):823-31.
55. Williams KM, Mella H, Lucas PJ, et al. Single cell analysis of complex thymus stromal cell populations: rapid thymic epithelia preparation characterizes radiation injury. Clin Transl Sci. 2009 Aug;2(4):279-85.

56. Lia G, Butera S, Evangelista A, et al. Long-term thymic function and reconstitution of the T cell compartment after T cell-replete haplo-identical allografting. Biology of Blood and Marrow Transplantation. 2019;25(3):S331.
57. Sarantopoulos S. Antibodies are back for thymic attack in cGVHD. Blood. 2016 May 5;127(18):2170-1.
58. Tivol E, Komorowski R, Drobyski WR. Emergent autoimmunity in graft-versus-host disease. Blood. 2005 Jun 15;105(12):4885-91.
59. Wu J, Yan Z, Schwartz DE, et al. Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. J Immunol. 2013 Apr 1;190(7):3590-9.
60. Sakoda Y, Hashimoto D, Asakura S, et al. Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. Blood. 2007 Feb 15;109(4):1756-64.
61. Flynn R, Du J, Veenstra RG, et al. Increased T follicular helper cells and germinal center B cells are required for cGVHD and bronchiolitis obliterans. Blood. 2014 Jun 19;123(25):3988-98.
62. Khoder A, Sarvaria A, Alsuliman A, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. Blood. 2014 Sep 25;124(13):2034-45.
63. Hu Y, He GL, Zhao XY, et al. Regulatory B cells promote graft-versus-host disease prevention and maintain graft-versus-leukemia activity following allogeneic bone marrow transplantation. Oncoimmunology. 2017;6(3):e1284721.
64. Kariminia A, Holtan SG, Ivison S, et al. Heterogeneity of chronic graft-versus-host disease biomarkers: association with CXCL10 and CXCR3+ NK cells. Blood. 2016 Jun 16;127(24):3082-91.
65. Du J, Paz K, Thangavelu G, et al. Invariant natural killer T cells ameliorate murine chronic GVHD by expanding donor regulatory T cells. Blood. 2017 Jun 8;129(23):3121-3125.
66. Zhang C, Todorov I, Zhang Z, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. Blood. 2006 Apr 1;107(7):2993-3001.
67. Zhao DM, Thornton AM, DiPaolo RJ, et al. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood. 2006 May 15;107(10):3925-32.
68. Allen JL, Fore MS, Wooten J, et al. B cells from patients with chronic GVHD are activated and primed for survival via BAFF-mediated pathways. Blood. 2012 Sep 20;120(12):2529-36.
69. Sarantopoulos S, Blazar BR, Cutler C, et al. B cells in chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2015 Jan;21(1):16-23.
70. Sarantopoulos S, Ritz J. Aberrant B-cell homeostasis in chronic GVHD. Blood. 2015 Mar 12;125(11):1703-7.
71. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, et al. The role of B cells in the pathogenesis of graft-versus-host disease. Blood. 2009 Dec 3;114(24):4919-27.
72. de Masson A, Bouaziz JD, Le Buanec H, et al. CD24(hi)CD27(+) and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. Blood. 2015 Mar 12;125(11):1830-9.
73. Le Huu D, Matsushita T, Jin G, et al. Donor-derived regulatory B cells are important for suppression of murine sclerodermatous chronic graft-versus-host disease. Blood. 2013;121(16):3274-3283.
74. Martires KJ, Baird K, Citrin DE, et al. Localization of sclerotic-type chronic graft-vs-host disease to sites of skin injury: potential insight into the mechanism of isomorphic and isotopic responses. Arch Dermatol. 2011 Sep;147(9):1081-6.
75. Wolff D, Greinix H, Lee SJ, et al. Biomarkers in chronic graft-versus-host disease: quo vadis? Bone Marrow Transplant. 2018 Jul;53(7):832-837.

76. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-214.
77. Noor F, Kaysen A, Wilmes P, et al. The gut microbiota and hematopoietic stem cell transplantation: challenges and potentials. Journal of innate immunity. 2019;11(5):405-415.
78. Staffas A, Burgos da Silva M, van den Brink MRM. The intestinal microbiota in allogeneic hematopoietic cell transplant and graft-versus-host disease. Blood. 2017;129(8):927-933.
*** This is a detailed review on the role of microbiome in allogeneic HSCT and GVHD.**
79. Holler E, Butzhammer P, Schmid K, et al. Metagenomic analysis of the stool microbiome in patients receiving allogeneic stem cell transplantation: loss of diversity is associated with use of systemic antibiotics and more pronounced in gastrointestinal graft-versus-host disease. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2014;20(5):640-645.
80. Peled JU, Gomes AL, Devlin SM, et al. Microbiota as predictor of mortality in allogeneic hematopoietic-cell transplantation. New England Journal of Medicine. 2020;382(9):822-834.
81. Shono Y, van den Brink MR. Gut microbiota injury in allogeneic haematopoietic stem cell transplantation. Nature Reviews Cancer. 2018;18(5):283.
82. Jenq RR, Taur Y, Devlin SM, et al. Intestinal Blautia is associated with reduced death from graft-versus-host disease. Biology of Blood and Marrow Transplantation. 2015;21(8):1373-1383.
83. Golob JL, Pergam SA, Srinivasan S, et al. Stool microbiota at neutrophil recovery is predictive for severe acute graft vs host disease after hematopoietic cell transplantation. Clinical Infectious Diseases. 2017;65(12):1984-1991.
84. Han L, Jin H, Zhou L, et al. Intestinal microbiota at engraftment influence acute graft-versus-host disease via the Treg/Th17 balance in allo-HSCT recipients. Frontiers in immunology. 2018;9:669.
85. Han L, Zhao K, Li Y, et al. A gut microbiota score predicting acute graft-versus-host disease following myeloablative allogeneic hematopoietic stem cell transplantation. American Journal of Transplantation. 2020;20(4):1014-1027.
86. Michonneau D, Latis E, Curis E, et al. Metabolomics analysis of human acute graft-versus-host disease reveals changes in host and microbiota-derived metabolites. Nature communications. 2019;10(1):5695-5695.
87. Riwes M, Reddy P. Microbial metabolites and graft versus host disease. American Journal of Transplantation. 2018;18(1):23-29.
88. Galloway-Peña JR, Peterson CB, Malik F, et al., editors. Fecal microbiome, metabolites, and stem cell transplant outcomes: A single-center pilot study. Open forum infectious diseases; 2019: Oxford University Press US.
89. Swimm A, Giver CR, DeFilipp Z, et al. Indoles derived from intestinal microbiota act via type I interferon signaling to limit graft-versus-host disease. Blood, The Journal of the American Society of Hematology. 2018;132(23):2506-2519.
90. Weber D, Oefner PJ, Hiergeist A, et al. Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome. Blood, The Journal of the American Society of Hematology. 2015;126(14):1723-1728.
91. Payen M, Nicolis I, Robin M, et al. Functional and phylogenetic alterations in gut microbiome are linked to graft-versus-host disease severity. Blood advances. 2020;4(9):1824-1832.
92. Markey KA, Schluter J, Gomes AL, et al. Microbe-derived short chain fatty acids butyrate and propionate are associated with protection from chronic GVHD. Blood Journal. 2020:blood. 2019003369.
93. Li Q, Zhai Z, Xu X, et al. Decrease of CD4(+)CD25(+) regulatory T cells and TGF-beta at early immune reconstitution is associated to the onset and severity of graft-versus-host

disease following allogeneic haematogenesis stem cell transplantation. Leuk Res. 2010 Sep;34(9):1158-68.

94. Magenau JM, Qin X, Tawara I, et al. Frequency of CD4(+)CD25(hi)FOXP3(+) regulatory T cells has diagnostic and prognostic value as a biomarker for acute graft-versus-host-disease. Biol Blood Marrow Transplant. 2010 Jul;16(7):907-14.

95. Zorn E, Kim HT, Lee SJ, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. Blood. 2005 Oct 15;106(8):2903-11.

96. Matsuoka K, Kim HT, McDonough S, et al. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. J Clin Invest. 2010 May;120(5):1479-93.

97. Miura Y, Thoburn CJ, Bright EC, et al. Association of Foxp3 regulatory gene expression with graft-versus-host disease. Blood. 2004 Oct 1;104(7):2187-93.

98. Budde H, Papert S, Maas JH, et al. Prediction of graft-versus-host disease: a biomarker panel based on lymphocytes and cytokines. Annals of hematology. 2017 Jul;96(7):1127-1133.

99. Huttunen P, Taskinen M, Siitonen S, et al. Impact of very early CD4(+) /CD8(+) T cell counts on the occurrence of acute graft-versus-host disease and NK cell counts on outcome after pediatric allogeneic hematopoietic stem cell transplantation. Pediatr Blood Cancer. 2015 Mar;62(3):522-8.

100. He FC, Holtan SG. Biomarkers in Graft-Versus-Host Disease: from Prediction and Diagnosis to Insights into Complex Graft/Host Interactions. Curr Hematol Malig Rep. 2018 Feb;13(1):44-52.

101. Greinix HT, Kuzmina Z, Weigl R, et al. CD19+CD21low B cells and CD4+CD45RA+CD31+ T cells correlate with first diagnosis of chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2015 Feb;21(2):250-8.

102. Ren HG, Adom D, Paczesny S. The search for drug-targetable diagnostic, prognostic and predictive biomarkers in chronic graft-versus-host disease. Expert Rev Clin Immunol. 2018 May;14(5):389-404.

103. Dander E, Balduzzi A, Zappa G, et al. Interleukin-17–producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem-cell transplantation. Transplantation. 2009;88(11):1261-1272.

104. Malard F, Bossard C, Brissot E, et al. Increased Th17/Treg ratio in chronic liver GVHD. Bone Marrow Transplant. 2014 Apr;49(4):539-44.

105. Forcade E, Paz K, Flynn R, et al. An activated Th17-prone T cell subset involved in chronic graft-versus-host disease sensitive to pharmacological inhibition. JCI Insight. 2017 Jun 15;2(12).

106. Li W, Liu L, Gomez A, et al. Proteomics analysis reveals a Th17-prone cell population in presymptomatic graft-versus-host disease. JCI Insight. 2016 May 5;1(6).

107. Forcade E, Kim HT, Cutler C, et al. Circulating T follicular helper cells with increased function during chronic graft-versus-host disease. Blood. 2016 May 19;127(20):2489-97.

108. Chen YB, McDonough S, Hasserjian R, et al. Expression of CD30 in patients with acute graft-versus-host disease. Blood. 2012 Jul 19;120(3):691-6.

109. Huenecke S, Cappel C, Esser R, et al. Development of Three Different NK Cell Subpopulations during Immune Reconstitution after Pediatric Allogeneic Hematopoietic Stem Cell Transplantation: Prognostic Markers in GvHD and Viral Infections. Front Immunol. 2017;8:109.

110. Sarantopoulos S, Stevenson KE, Kim HT, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. Blood. 2009 Apr 16;113(16):3865-74.

111. Jacobson CA, Turki AT, McDonough SM, et al. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. Biol Blood Marrow Transplant. 2012 Apr;18(4):565-74.

112. Rozmus J, Kariminia A, Abdossamadi S, et al. Comprehensive B Cell Phenotyping Profile for Chronic Graft-versus-Host Disease Diagnosis. Biol Blood Marrow Transplant. 2019 Mar;25(3):451-458.
113. Stikvoort A, Chen Y, Radestad E, et al. Combining Flow and Mass Cytometry in the Search for Biomarkers in Chronic Graft-versus-Host Disease. Front Immunol. 2017;8:717.
114. Kuzmina Z, Greinix HT, Weigl R, et al. Significant differences in B-cell subpopulations characterize patients with chronic graft-versus-host disease-associated dysgammaglobulinemia. Blood. 2011 Feb 17;117(7):2265-74.
115. Kuzmina Z, Krenn K, Petkov V, et al. CD19(+)CD21(low) B cells and patients at risk for NIH-defined chronic graft-versus-host disease with bronchiolitis obliterans syndrome. Blood. 2013 Mar 7;121(10):1886-95.
116. Greinix HT, Pohlreich D, Kouba M, et al. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27+ B cells identify patients with active chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2008 Feb;14(2):208-19.
117. Bohmann EM, Fehn U, Holler B, et al. Altered immune reconstitution of B and T cells precedes the onset of clinical symptoms of chronic graft-versus-host disease and is influenced by the type of onset. Annals of hematology. 2017 Feb;96(2):299-310.
118. Lawitschka A, Gueclue ED, Januszko A, et al. National Institutes of Health-Defined Chronic Graft-vs.-Host Disease in Pediatric Hematopoietic Stem Cell Transplantation Patients Correlates With Parameters of Long-Term Immune Reconstitution. Front Immunol. 2019;10:1879.
119. Kuzmina Z, Greinix HT, Knobler R, et al. Proportions of immature CD19+CD21- B lymphocytes predict the response to extracorporeal photopheresis in patients with chronic graft-versus-host disease. Blood. 2009 Jul 16;114(3):744-6.
120. D'Orsogna LJ, Wright MP, Krueger RG, et al. Allogeneic hematopoietic stem cell transplantation recipients have defects of both switched and igm memory B cells. Biol Blood Marrow Transplant. 2009 Jul;15(7):795-803.
121. Schultz KR, Kariminia A, Ng B, et al. Immune profile differences between chronic GVHD and late acute GVHD: results of the ABLE/PBMTC 1202 studies. Blood. 2020 Apr 9;135(15):1287-1298.
122. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. The Journal of experimental medicine. 1994 Sep 1;180(3):1097-106.
123. Gumperz JE, Miyake S, Yamamura T, et al. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. The Journal of experimental medicine. 2002 Mar 4;195(5):625-36.
124. Lan F, Zeng D, Higuchi M, et al. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. Biol Blood Marrow Transplant. 2003 Jun;9(6):355-63.
125. Leveson-Gower DB, Olson JA, Sega EI, et al. Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. Blood. 2011 Mar 17;117(11):3220-9.
126. Pillai AB, George TI, Dutt S, et al. Host natural killer T cells induce an interleukin-4-dependent expansion of donor CD4+CD25+Foxp3+ T regulatory cells that protects against graft-versus-host disease. Blood. 2009 Apr 30;113(18):4458-67.
127. Hashimoto D, Asakura S, Miyake S, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. J Immunol. 2005 Jan 1;174(1):551-6.
128. Schneidawind D, Pierini A, Alvarez M, et al. CD4+ invariant natural killer T cells protect from murine GVHD lethality through expansion of donor CD4+CD25+FoxP3+ regulatory T cells. Blood. 2014 Nov 20;124(22):3320-8.

129. Hongo D, Tang X, Dutt S, et al. Interactions between NKT cells and Tregs are required for tolerance to combined bone marrow and organ transplants. Blood. 2012 Feb 9;119(6):1581-9.
130. Haraguchi K, Takahashi T, Hiruma K, et al. Recovery of Valpha24+ NKT cells after hematopoietic stem cell transplantation. Bone Marrow Transplant. 2004 Oct;34(7):595-602.
131. Rubio MT, Moreira-Teixeira L, Bachy E, et al. Early posttransplantation donor-derived invariant natural killer T-cell recovery predicts the occurrence of acute graft-versus-host disease and overall survival. Blood. 2012 Sep 6;120(10):2144-54.
132. Chaidos A, Patterson S, Szydlo R, et al. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. Blood. 2012 May 24;119(21):5030-6.
133. Malard F, Labopin M, Chevallier P, et al. Larger number of invariant natural killer T cells in PBSC allografts correlates with improved GVHD-free and progression-free survival. Blood. 2016 Apr 7;127(14):1828-35.
134. Rubio MT, Bouillie M, Bouazza N, et al. Pre-transplant donor CD4(-) invariant NKT cell expansion capacity predicts the occurrence of acute graft-versus-host disease. Leukemia. 2017 Apr;31(4):903-912.
135. Luft T, Dietrich S, Falk C, et al. Steroid-refractory GVHD: T-cell attack within a vulnerable endothelial system. Blood. 2011 Aug 11;118(6):1685-92.
136. Jiang S, Walker L, Afentoulis M, et al. Transplanted human bone marrow contributes to vascular endothelium. Proc Natl Acad Sci U S A. 2004 Nov 30;101(48):16891-6.
137. Willemze AJ, Bakker AC, von dem Borne PA, et al. The effect of graft-versus-host disease on skin endothelial and epithelial cell chimerism in stem-cell transplant recipients. Transplantation. 2009 Apr 15;87(7):1096-101.
138. Penack O, Socie G, van den Brink MR. The importance of neovascularization and its inhibition for allogeneic hematopoietic stem cell transplantation. Blood. 2011 Apr 21;117(16):4181-9.
139. Almici C, Skert C, Verardi R, et al. Changes in circulating endothelial cells count could become a valuable tool in the diagnostic definition of acute graft-versus-host disease. Transplantation. 2014 Oct 15;98(7):706-12.
140. Almici C, Skert C, Bruno B, et al. Circulating endothelial cell count: a reliable marker of endothelial damage in patients undergoing hematopoietic stem cell transplantation. Bone Marrow Transplant. 2017 Dec;52(12):1637-1642.
141. Almici C, Neva A, Skert C, et al. Counting circulating endothelial cells in allo-HSCT: an ad hoc designed polychromatic flowcytometry-based panel versus the CellSearch System. Scientific reports. 2019 Jan 14;9(1):87.
142. Tétreault N, De Guire V. miRNAs: their discovery, biogenesis and mechanism of action. Clinical biochemistry. 2013;46(10-11):842-845.
143. Tomuleasa C, Fuji S, Cucuianu A, et al. MicroRNAs as biomarkers for graft-versus-host disease following allogeneic stem cell transplantation. Annals of hematology. 2015;94(7):1081-1092.
144. Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. British journal of haematology. 2008;141(5):672-675.
145. Newmarch M, Kostantin E, Tsongalis G, et al. MicroRNAs in graft-versus-host disease: a review of the latest data. Bone Marrow Transplantation. 2019:1-7.
146. Peltier D, Reddy P. Non-coding RNA mediated regulation of allogeneic T cell responses after hematopoietic transplantation. Frontiers in immunology. 2018;9:1110.
147. Lee C-W, Wohlan K, Dallmann I, et al. miR-181a expression in donor T cells modulates graft-versus-host disease after allogeneic bone marrow transplantation. The Journal of Immunology. 2016;196(9):3927-3934.

148. Sang W, Zhang C, Zhang D, et al. MicroRNA-181a, a potential diagnosis marker, alleviates acute graft versus host disease by regulating IFN-γ production. American journal of hematology. 2015;90(11):998-1007.
149. Ward PA, Fattahi F, Bosmann M. New insights into molecular mechanisms of immune complex-induced injury in lung. Frontiers in immunology. 2016;7:86.
150. Stickel N, Prinz G, Pfeifer D, et al. MiR-146a regulates the TRAF6/TNF-axis in donor T cells during GVHD. Blood, The Journal of the American Society of Hematology. 2014;124(16):2586-2595.
151. Alivernini S, Gremese E, McSharry C, et al. MicroRNA-155—at the critical interface of innate and adaptive immunity in arthritis. Frontiers in immunology. 2018;8:1932.
152. Ranganathan P. THE DISTILLERY.
153. Xie LN, Zhou F, Liu XM, et al. Serum micro RNA 155 is increased in patients with acute graft-versus-host disease. Clinical transplantation. 2014;28(3):314-323.
154. Lendrem C, Greinix H, Dickinson A. Crossland RE, Norden J, Juric MK, Green K, Pearce KF. 2017.
155. Atarod S, Norden J, Bibby LA, et al. Differential Microrna expression levels in cutaneous acute graft-Versus-host Disease. Frontiers in Immunology. 2018;9:1485.
156. Xiao B, Wang Y, Li W, et al. Plasma microRNA signature as a noninvasive biomarker for acute graft-versus-host disease. Blood. 2013;122(19):3365-3375.
157. Crossland RE, Norden J, Collin M, et al. Urinary Micrornas MiR-377, MiR-423, MiR-93 and MiR-199 As Biomarkers for Graft Versus Host Disease. American Society of Hematology Washington, DC; 2014.
158. Greinix H, Dickinson A. Crossland RE, Norden J, Juric MK, Pearce KF, Lendrem C, Bibby LA, Collin M. 2017.
159. Zhang C, Bai N, Huang W, et al. The predictive value of selected serum microRNAs for acute GVHD by TaqMan MicroRNA arrays. Annals of hematology. 2016;95(11):1833-1843.
160. Callari M, Tiberio P, De Cecco L, et al. Feasibility of circulating miRNA microarray analysis from archival plasma samples. Analytical biochemistry. 2013;437(2):123-125.
161. Mason TE, Ricks-Santi L, Chen W, et al. Association of CD14 variant with prostate cancer in African American men. The Prostate. 2010;70(3):262-269.
162. García-Silva S, Benito-Martín A, Sánchez-Redondo S, et al. Use of extracellular vesicles from lymphatic drainage as surrogate markers of melanoma progression and BRAFV600E mutation. Journal of Experimental Medicine. 2019;216(5):1061-1070.
163. Vagner T, Spinelli C, Minciacchi VR, et al. Large extracellular vesicles carry most of the tumour DNA circulating in prostate cancer patient plasma. J Extracell Vesicles. 2018;7(1):1505403.
164. Johnson A. Role of Extracellular Vesicles in Development of Antiandrogen Resistance in Prostate Cancer: Tulane University; 2018.
165. Yang S, Li X. Recent advances in extracellular vesicles enriched with non-coding RNAs related to cancers. Genes & diseases. 2018;5(1):36-42.
166. Atay S, Wilkey DW, Milhem M, et al. Insights into the proteome of gastrointestinal stromal tumors-derived exosomes reveals new potential diagnostic biomarkers. Molecular & Cellular Proteomics. 2018;17(3):495-515.
167. Dourado MR, Korvala J, Åström P, et al. Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. J Extracell Vesicles. 2019;8(1):1578525.
168. Allenson K, Castillo J, San Lucas F, et al. High prevalence of mutant KRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients. Annals of Oncology. 2017;28(4):741-747.

169. Castillo J, Bernard V, San Lucas FA, et al. Surfaceome profiling enables isolation of cancer-specific exosomal cargo in liquid biopsies from pancreatic cancer patients. Annals of Oncology. 2018 2018/01/01/;29(1):223-229.
170. Maas SLN, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. Trends Cell Biol. 2017;27(3):172-188.
171. Yáñez-Mó M, Siljander PRM, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:27066-27066.
172. Wu Q, Chen H, Fang J, et al. Elevated Fas/FasL system and endothelial cell microparticles are involved in endothelial damage in acute graft-versus-host disease: a clinical analysis. Leukemia research. 2012;36(3):275-280.
173. Lia G, Brunello L, Bruno S, et al. Extracellular vesicles as potential biomarkers of acute graft-vs-host disease. Leukemia. 2018;32(3):765-773.
174. Brunello L, Lia G, Bruno S, et al. Biomarkers of Acute Graft-Versus-Host Disease: Surface Antigens and Micro Rnas in Extracellular Vesicles. Biology of Blood and Marrow Transplantation. 2019;25(3):S232.
175. Zhang R, Wang X, Hong M, et al. Endothelial microparticles delivering microRNA-155 into T lymphocytes are involved in the initiation of acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation. Oncotarget. 2017;8(14):23360.
176. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. Nat Immunol. 2001 Feb;2(2):102-7.
177. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nat Immunol. 2001 Feb;2(2):123-8.
178. Ali AM, DiPersio JF, Schroeder MA. The Role of Biomarkers in the Diagnosis and Risk Stratification of Acute Graft-versus-Host Disease: A Systematic Review. Biol Blood Marrow Transplant. 2016 Sep;22(9):1552-1564.
179. August KJ, Chiang KY, Bostick RM, et al. Biomarkers of immune activation to screen for severe, acute GVHD. Bone Marrow Transplant. 2011 Apr;46(4):601-4.
180. Busca A, Locatelli F, Marmont F, et al. Recombinant human soluble tumor necrosis factor receptor fusion protein as treatment for steroid refractory graft-versus-host disease following allogeneic hematopoietic stem cell transplantation. Am J Hematol. 2007 Jan;82(1):45-52.
181. Levine JE, Paczesny S, Mineishi S, et al. Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. Blood. 2008 Feb 15;111(4):2470-5.
182. Malard F, Huang X-J, Sim JPY. Treatment and unmet needs in steroid-refractory acute graft-versus-host disease. Leukemia. 2020 2020/05/01;34(5):1229-1240.
183. Choi SW, Kitko CL, Braun T, et al. Change in plasma tumor necrosis factor receptor 1 levels in the first week after myeloablative allogeneic transplantation correlates with severity and incidence of GVHD and survival. Blood. 2008 Aug 15;112(4):1539-42.
184. Kitko CL, Paczesny S, Yanik G, et al. Plasma elevations of tumor necrosis factor-receptor-1 at day 7 postallogeneic transplant correlate with graft-versus-host disease severity and overall survival in pediatric patients. Biol Blood Marrow Transplant. 2008 Jul;14(7):759-65.
185. Hill GR, Teshima T, Rebel VI, et al. The p55 TNF-alpha receptor plays a critical role in T cell alloreactivity. J Immunol. 2000 Jan 15;164(2):656-63.
186. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013 Jan 24;38(1):13-25.
187. Zhang L, Yu J, Wei W. Advance in targeted immunotherapy for graft-versus-host disease. Frontiers in immunology. 2018;9:1087.
188. Miyamoto T, Akashi K, Hayashi S, et al. Serum concentration of the soluble interleukin-2 receptor for monitoring acute graft-versus-host disease. Bone Marrow Transplant. 1996 Feb;17(2):185-90.

189. Foley R, Couban S, Walker I, et al. Monitoring soluble interleukin-2 receptor levels in related and unrelated donor allogenic bone marrow transplantation. Bone Marrow Transplant. 1998 Apr;21(8):769-73.
190. Tedesco D, Haragsim L. Cyclosporine: a review. Journal of transplantation. 2012;2012.
191. Griesenauer B, Paczesny S. The ST2/IL-33 axis in immune cells during inflammatory diseases. Frontiers in immunology. 2017;8:475.
*** This is a detailed review on ST2/IL-33 signalling pathway and its role in the context of different inflammatory diseases.**
192. Castor MG, Pinho V, Teixeira MM. The role of chemokines in mediating graft versus host disease: opportunities for novel therapeutics. Front Pharmacol. 2012;3:23.
193. Murai M, Yoneyama H, Harada A, et al. Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. J Clin Invest. 1999 Jul;104(1):49-57.
194. Palmer LA, Sale GE, Balogun JI, et al. Chemokine receptor CCR5 mediates alloimmune responses in graft-versus-host disease. Biol Blood Marrow Transplant. 2010 Mar;16(3):311-9.
195. Hutter G, Neumann M, Nowak D, et al. The effect of the CCR5-delta32 deletion on global gene expression considering immune response and inflammation. J Inflamm (Lond). 2011 Oct 26;8:29.
196. Bogunia-Kubik K, Duda D, Suchnicki K, et al. CCR5 deletion mutation and its association with the risk of developing acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Haematologica. 2006 Dec;91(12):1628-34.
197. Ma Q, Gooley TA, Storb RF. CCR5 expression on cells from HLA-matched unrelated marrow donors and graft-versus-host disease. Biol Blood Marrow Transplant. 2010 Jan;16(1):132-3.
198. Moy RH, Huffman AP, Richman LP, et al. Clinical and immunologic impact of CCR5 blockade in graft-versus-host disease prophylaxis. Blood. 2017 Feb 16;129(7):906-916.
199. Reshef R, Ganetsky A, Acosta EP, et al. Extended CCR5 Blockade for Graft-versus-Host Disease Prophylaxis Improves Outcomes of Reduced-Intensity Unrelated Donor Hematopoietic Cell Transplantation: A Phase II Clinical Trial. Biol Blood Marrow Transplant. 2019 Mar;25(3):515-521.
200. Ahmed SS, Wang XN, Norden J, et al. Identification and validation of biomarkers associated with acute and chronic graft versus host disease. Bone marrow transplantation. 2015;50(12):1563-1571.
201. Chen YB, Cutler CS. Biomarkers for acute GVHD: can we predict the unpredictable? Bone Marrow Transplant. 2013 Jun;48(6):755-60.
202. Kitko CL, Levine JE, Storer BE, et al. Plasma CXCL9 elevations correlate with chronic GVHD diagnosis. Blood. 2014;123(5):786-793.
203. Yu J, Storer BE, Kushekhar K, et al. Biomarker panel for chronic graft-versus-host disease. Journal of Clinical Oncology. 2016;34(22):2583.
204. Ahmed S, Wang X, Norden J, et al. Erratum: Identification and validation of biomarkers associated with acute and chronic graft versus host disease. Bone Marrow Transplantation. 2016;51(6):890-890.
205. Reichenbach DK, Schwarze V, Matta BM, et al. The IL-33/ST2 axis augments effector T-cell responses during acute GVHD. Blood, The Journal of the American Society of Hematology. 2015;125(20):3183-3192.
206. Barrett AJ. Transplant biomarkers ready for the clinic? Blood. 2017;129(2):137-139.
207. Westekemper H, Meller S, Citak S, et al. Differential chemokine expression in chronic GVHD of the conjunctiva. Bone marrow transplantation. 2010;45(8):1340-1346.

208. Fujii H, Cuvelier G, She K, et al. Biomarkers in newly diagnosed pediatric-extensive chronic graft-versus-host disease: a report from the Children's Oncology Group. Blood, The Journal of the American Society of Hematology. 2008;111(6):3276-3285.
209. Pratt L, Liu Y, Ugarte-Torres A, et al. IL15 levels on day 7 after hematopoietic cell transplantation predict chronic GVHD. Bone marrow transplantation. 2013;48(5):722-728.
210. Skert C, Damiani D, Michelutti A, et al. Kinetics of Th1/Th2 cytokines and lymphocyte subsets to predict chronic GVHD after allo-SCT: results of a prospective study. Bone Marrow Transplant. 2009 Dec;44(11):729-37.
211. Kobayashi S, Imamura M, Hashino S, et al. Clinical relevance of serum soluble interleukin-2 receptor levels in acute and chronic graft-versus-host disease. Leukemia & lymphoma. 1997;28(1-2):159-169.
212. Zhao X-S, Huang X-J. Seeking biomarkers for acute graft-versus-host disease: where we are and where we are heading? Biomarker Research. 2019;7(1):1-10.
213. Paczesny S, Krijanovski OI, Braun TM, et al. A biomarker panel for acute graft-versus-host disease. Blood, The Journal of the American Society of Hematology. 2009;113(2):273-278.
*** This study uses antibody microarrays against 120 proteins to screen aGVHD biomarkers in peripheral blood.**
214. Harris AC, Ferrara JL, Braun TM, et al. Plasma biomarkers of lower gastrointestinal and liver acute GVHD. Blood. 2012 Mar 22;119(12):2960-3.
215. Lugt PR, Chin A, Zhang Q, et al. Regenerating islet-derived 3 alpha is a biomarker of gastrointestinal. 2011.
216. Paczesny S, Braun TM, Levine JE, et al. Elafin Is a Biomarker of Graft-Versus-Host Disease of the Skin. Science Translational Medicine. 2010;2(13):13ra2.
217. Ferrara JL, Harris AC, Greenson JK, et al. Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease. Blood. 2011 Dec 15;118(25):6702-8.
218. Levine JE, Logan BR, Wu J, et al. Acute graft-versus-host disease biomarkers measured during therapy can predict treatment outcomes: a Blood and Marrow Transplant Clinical Trials Network study. Blood, The Journal of the American Society of Hematology. 2012;119(16):3854-3860.
219. Ponce DM, Hilden P, Mumaw C, et al. High day 28 ST2 levels predict for acute graft-versus-host disease and transplant-related mortality after cord blood transplantation. Blood. 2015 Jan 1;125(1):199-205.
220. Vander Lugt MT, Braun TM, Hanash S, et al. ST2 as a marker for risk of therapy-resistant graft-versus-host disease and death. N Engl J Med. 2013 Aug 8;369(6):529-39.
221. Hartwell MJ, Ozbek U, Holler E, et al. An early-biomarker algorithm predicts lethal graft-versus-host disease and survival. JCI Insight. 2017 Feb 9;2(3):e89798.
**** This study proposes a predictive algorithm based on a plasmatic panel that is the most advanced in clinical development.**
222. Solán L, Kwon M, Carbonell D, et al. ST2 and REG3α as predictive biomarkers after haploidentical stem cell transplantation using post-transplantation high-dose cyclophosphamide. Frontiers in immunology. 2019;10:2338.
223. Srinagesh HK, Ferrara JLM. MAGIC biomarkers of acute graft-versus-host disease: Biology and clinical application. Best Practice & Research Clinical Haematology. 2019 2019/12/01/;32(4):101111.
224. Levine JE, Braun TM, Harris AC, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. The Lancet Haematology. 2015;2(1):e21-e29.
225. McDonald GB, Tabellini L, Storer BE, et al. Plasma biomarkers of acute GVHD and nonrelapse mortality: predictive value of measurements before GVHD onset and treatment. Blood, The Journal of the American Society of Hematology. 2015;126(1):113-120.

226. Abu Zaid M, Wu J, Wu C, et al. Plasma biomarkers of risk for death in a multicenter phase 3 trial with uniform transplant characteristics post–allogeneic HCT. Blood, The Journal of the American Society of Hematology. 2017;129(2):162-170.

227. Etra A, Gergoudis S, Morales G, et al. Comparison of GVHD biomarker algorithms for predicting lethal GVHD and non-relapse mortality. Biology of Blood and Marrow Transplantation. 2019;25(3):S53-S54.

228. Weissinger E, Metzger J, Dobbelstein C, et al. Proteomic peptide profiling for preemptive diagnosis of acute graft-versus-host disease after allogeneic stem cell transplantation. Leukemia. 2014;28(4):842-852.

229. Devic I, Shi M, Schubert MM, et al. Proteomic analysis of saliva from patients with oral chronic graft-versus-host disease. Biology of Blood and Marrow Transplantation. 2014;20(7):1048-1055.

230. Chiusolo P, Giammarco S, Fanali C, et al. Salivary proteomic analysis and acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Biology of Blood and Marrow Transplantation. 2013;19(6):888-892.

231. Kennedy GA, Varelias A, Vuckovic S, et al. Addition of interleukin-6 inhibition with tocilizumab to standard graft-versus-host disease prophylaxis after allogeneic stem-cell transplantation: a phase 1/2 trial. Lancet Oncol. 2014 Dec;15(13):1451-1459.

232. Mohamed AJ, Yu L, Bäckesjö CM, et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. Immunol Rev. 2009 Mar;228(1):58-73.

233. Dubovsky JA, Beckwith KA, Natarajan G, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood. 2013 Oct 10;122(15):2539-49.

234. Crossland RE, Norden J, Juric MK, et al. Expression of Serum microRNAs is Altered During Acute Graft-versus-Host Disease. Front Immunol. 2017;8:308.
*** This study profiles expression of 799 mature microRNAs in patient serum identifying altered expression at aGVHD diagnosis.**

235. Crossland RE, Norden J, Kralj Juric M, et al. Serum and Extracellular Vesicle MicroRNAs miR-423, miR-199, and miR-93* As Biomarkers for Acute Graft-versus-Host Disease. Front Immunol. 2017;8:1446.

Table 1.

BIOMARKER	LEVELS	aGVHD	cGVHD	*diagnostic*	*prognostic*	*predictive*	*clinical trial*
SNPs		[18-20]	[19]		[18-20]		
microbiota	impaired bacterial species	[83-85]			[83-85]		
SCFAs (butyrate)	reduced	[88,91]			[88,91]		
Cellular biomarkers							
Tregs	reduced	[93,94]	[93,95-97]	[95-97]	[93,95-97]	[94]	
CD4+CD45RA+CD31+	increased		[101]	[101]	[101]		
CD4+/CD8+	increased	[98-100]			[98-100]		
Th17	increased		[103,104]	[103,104]			
CD4+CD146+CCR5+	increased	[106]	[105]	[105]	[106]		
cTFH	reduced		[107]	[107]			
CD56^{bright} NK cells	reduced	[109]	[64,210]	[64]	[109,210]		
CD8+CD30+ T cells	expressed	[108]		[108]		[108]	
BAFF/B cells	increased		[110-112]	[110-112]			
CD19+CD21^{low} B cells	increased		[112,115, 117,118]	[112,115,117, 118]	[101,112]	[118,119]	
CD27+ memory B cells	reduced		[114,116, 120]	[114,116,120]	[118]		
iNKT	reduced	[130,133]		[130]	[133]		
iNKT/T cells	reduced	[131]			[131]		
CD4^{-}iNKT graft content	protective if increased	[132]		[132]	[132]		
CECs	reduced at engraftment	[139,140]			[139,140]	[139,140]	
miRNAs and EVs							
miR146a	reduced	[155,234]		[234]	[155]		
miR155	increased	[153,155]		[153]	[155]		
miR181a	reduced	[148]		[148,234]			
miR423, miR199a-3p, miR93, miR377	increased	[156,235]		[156,157,235]	[156,157,235]		
miR26b, miR374a, miR489, miR28-5p, miR671-3p	increased	[159]		[159]			
EVs membrane antigens (CD146, CD31, CD140a)	increased	[173]			[173]		
Cytokines and Chemokines							
sIL-2Rα	increased	[188]			[188]		
sST2	increased	[219,220]			[219]	[220]	
sST2, CXCL9, OPN, MMP3	increased		[203]	[203]	[203]		
CCR5Δ32 allele	protective if present	[196,197]			[196,197]		
CXCL9	Increased		[202,203]	[202,203]			
CXCL10, CXCL11	increased		[204]	[204]			
sBAFF	increased		[208]	[208]		[208]	
IL-15	reduced		[209]		[209]		
Proteomics							
IL-2Rα, HGF, IL-8, TNFR	increased	[213,218]		[213]	[218]	[218]	NCT00224874
REG3α, elafin, KRT18	increased	[214,217, 218]		[214]	[214,217,218]	[217,218]	NCT00224874
REG3α, ST2, TNFR1, IL-2Rα	increased	[221,222, 224]			[221,222,224]	[221,223, 224,227]	NCT02806947
aGVHD_MS17	variable	[228]	[229]	[228,229]	[228]		

List of biomarkers involved in acute and chronic Graft-versus-host disease, according to their diagnostic, prognostic or predictive value.

Abbreviations: aGVHD=acute graft-versus-host disease; cGVHD=chronic graft-versus-host disease; miRNAs=microRNAs; EVs=extracellular vesicles; SNPs=single-nucleotide polymorphisms; SCFAs=short-chain fatty acids; Tregs=regulatory T cells; Th17=T helper 17 cells; cTFH=follicular helper T cells; BAFF=B-cell activating factor; iNKT=invariant natural killer T cells; CECs=circulating endothelial cells; IL-2Rα=interleukin-2 receptor alpha-chain; sST2=soluble suppressor of tumorigenicity 2; CXCL9=C-X-C motif chemokine ligand 9; OPN=osteopontin; MMP3=matrix metalloproteinase 3; IL-15=interleukin-15; HGF=hepatocyte growth factor; IL-8=interleukin-8; TNFR1=tumor necrosis factor receptor 1; REG3α=regenerating islet-derived protein 3α; KRT18=cytokeratine-18 fragments

Figure 1

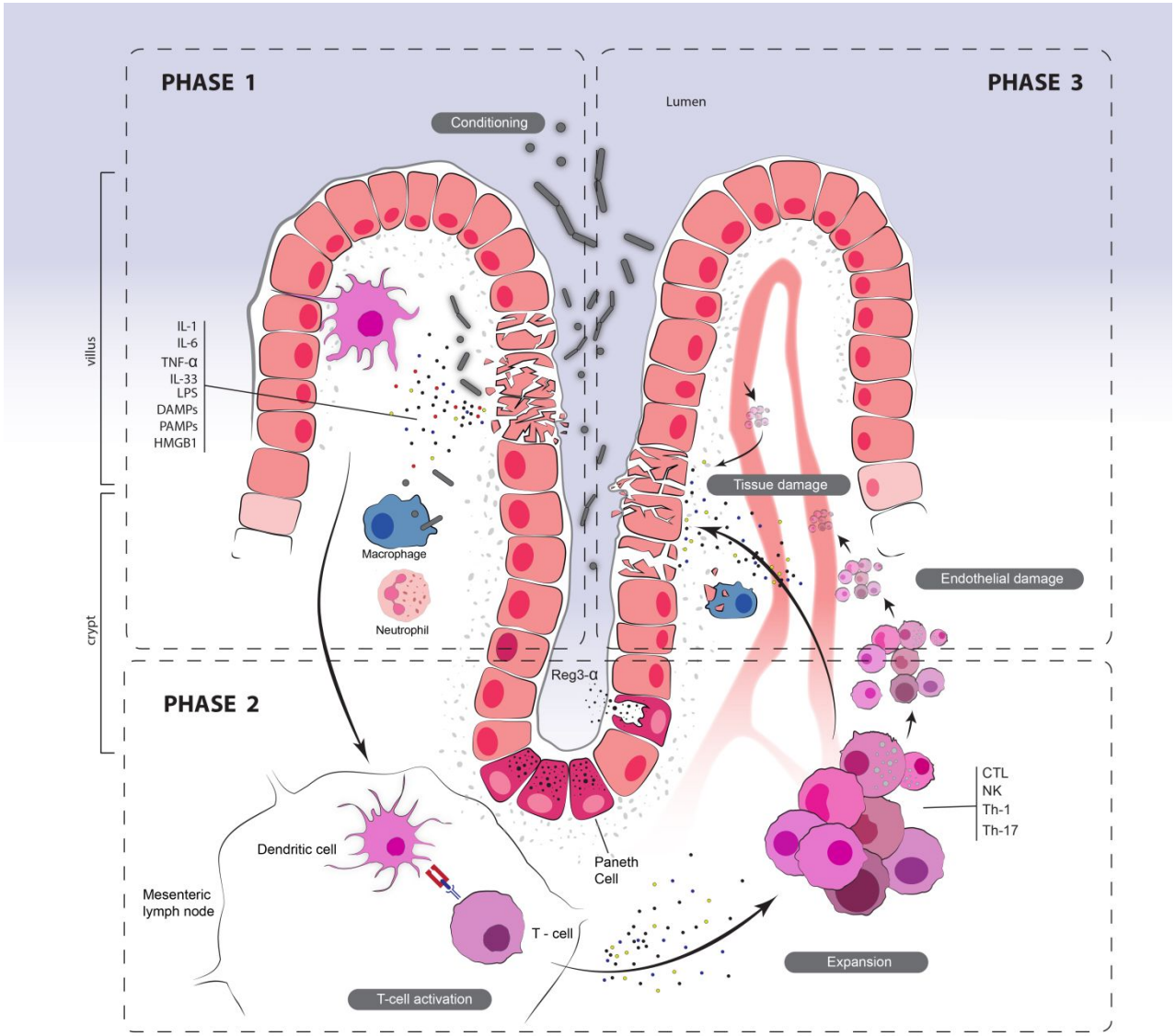
PHASE 1
PHASE 3
Lumen
Conditioning
villus
crypt
IL-1
IL-6
TNF-α
IL-33
LPS
DAMPs
PAMPs
HMGB1
Macrophage
Neutrophil
Tissue damage
Endothelial damage
Reg3-α
PHASE 2
Dendritic cell
Mesenteric lymph node
T - cell
Paneth Cell
CTL
NK
Th-1
Th-17
Expansion
T-cell activation

Figure 1 legend

The pathophysiology of acute GVHD (aGVHD) has been historically divided into three distinct phases: (1) the first step involves conditioning-induced tissue damage and subsequent release of inflammatory cytokines, including TNF-α, IL-6, IL-1α, and alarmins such as IL-33. In addition, loss of diversity in intestinal microbiota leads to loss of homeostasis with host immune system; (2) in the second phase, both host and donor derived antigen presenting cells (APCs) activate and expand alloreactive T cells. The inflammatory response is partly mediated by innate immune effectors (neutrophils, phagocytes, NK cells) stimulated by translocation through the damaged intestinal mucosa of lipopolysaccharide (LPS), damage associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs); (3) in the third phase, pathogenic effector cells and inflammatory mediators lead to the disease. Activated T cells migrate to target organs where they cause tissue damage and produce proinflammatory cytokines attracting other cellular effectors. Of note, damage of Paneth cells induces release of REG3α into bloodstream.

BIOMARKER	LEVELS	aGVHD	cGVHD	*diagnostic*	*prognostic*	*predictive*	*clinical trial*
SNPs		[18-20]	[19]		[18-20]		
microbiota	impaired bacterial species	[83-85]			[83-85]		
SCFAs (butyrate)	reduced	[88,91]			[88,91]		
Cellular biomarkers							
Tregs	reduced	[93,94]	[93,95-97]	[95-97]	[93,95-97]	[94]	
CD4+CD45RA+CD31+	increased		[101]	[101]	[101]		
CD4+/CD8+	increased	[98-100]			[98-100]		
Th17	increased		[103,104]	[103,104]			
CD4+CD146+CCR5+	increased	[106]	[105]	[105]	[106]		
cTFH	reduced		[107]	[107]			
CD56^{bright} NK cells	reduced	[109]	[64,210]	[64]	[109,210]		
CD8+CD30+ T cells	expressed	[108]		[108]		[108]	
BAFF/B cells	increased		[110-112]	[110-112]			
CD19+CD21^{low} B cells	increased		[112,115, 117,118]	[112,115,117, 118]	[101,112]	[118,119]	
CD27+ memory B cells	reduced		[114,116, 120]	[114,116,120]	[118]		
iNKT	reduced	[130,133]		[130]	[133]		
iNKT/T cells	reduced	[131]			[131]		
CD4^{-}iNKT graft content	protective if increased	[132]		[132]	[132]		
CECs	reduced at engraftment	[139,140]			[139,140]	[139,140]	
miRNAs and EVs							
miR146a	reduced	[155,234]		[234]	[155]		
miR155	increased	[153,155]		[153]	[155]		
miR181a	reduced	[148]		[148,234]			
miR423, miR199a-3p, miR93, miR377	increased	[156,235]		[156,157,235]	[156,157,235]		
miR26b, miR374a, miR489, miR28-5p, miR671-3p	increased	[159]		[159]			
EVs membrane antigens (CD146, CD31, CD140a)	increased	[173]			[173]		
Cytokines and Chemokines							
sIL-2Rα	increased	[188]			[188]		
sST2	increased	[219,220]			[219]	[220]	
sST2, CXCL9, OPN, MMP3	increased		[203]	[203]	[203]		
CCR5Δ32 allele	protective if present	[196,197]			[196,197]		
CXCL9	Increased		[202,203]	[202,203]			
CXCL10, CXCL11	increased		[204]	[204]			
sBAFF	increased		[208]	[208]		[208]	
IL-15	reduced		[209]		[209]		
Proteomics							
IL-2Rα, HGF, IL-8, TNFR	increased	[213,218]		[213]	[218]	[218]	NCT00224874
REG3α, elafin, KRT18	increased	[214,217, 218]		[214]	[214,217,218]	[217,218]	NCT00224874
REG3α, ST2, TNFR1, IL-2Rα	increased	[221,222, 224]			[221,222,224]	[221,223, 224,227]	NCT02806947
aGVHD_MS17	variable	[228]	[229]	[228,229]	[228]		

List of biomarkers involved in acute and chronic Graft-versus-host disease, according to their diagnostic, prognostic or predictive value.

Abbreviations: aGVHD=acute graft-versus-host disease; cGVHD=chronic graft-versus-host disease; miRNAs=microRNAs; EVs=extracellular vesicles; SNPs=single-nucleotide polymorphisms; SCFAs=short-chain fatty acids; Tregs=regulatory T cells; Th17=T helper 17 cells; cTFH=follicular helper T cells; BAFF=B-cell activating factor; iNKT=invariant natural killer T cells; CECs=circulating endothelial cells; IL-2Rα=interleukin-2 receptor alpha-chain; sST2=soluble suppressor of tumorigenicity 2; CXCL9=C-X-C motif chemokine ligand 9; OPN=osteopontin; MMP3=matrix metalloproteinase 3; IL-15=interleukin-15; HGF=hepatocyte growth factor; IL-8=interleukin-8; TNFR1=tumor necrosis factor receptor 1; REG3α=regenerating islet-derived protein 3α; KRT18=cytokeratine-18 fragments