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Review

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

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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.

Key words

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

Article highlights box

- 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 (IL1RL1) 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 38% 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 [48,57,58]. 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 ≥ 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. 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 miR518 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 GVHD in several studies [64,110,202-204]. In addition to its correlation with aGVHD [205], ST2 possesses 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 NCT02248874) 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 last 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.

5. 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.

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Declaration of interest

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BIOMARKER	LEVELS	aGVHD	cGVHD	*diagnostic*	*prognostic*	*predictive*	*clinical trial*
SNPs		[18-20]	[19]		[18-20]		
microbiota	impaired bacterial	[83-85]			[83-85]		
SCFAs (butyrate)	reduced	[88,91]			[88,91]		
Cellular biomarkers							
Tregs	reduced	[93,94]	[93,95-]	[95-97]	[93,95-97]	[94]	
CD4+CD45RA+CD31	increased		[101]	[101]	[101]		
CD4+/CD8+	increased	[98-			[98-100]		
Th17	increased		[103,10	[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-	[110-112]			
CD19+CD21^{low} B	increased		[112,11	[112,115,1	[101,112]	[118,11	
CD27+ memory B	reduced		[114,11	[114,116,1	[118]		
iNKT	reduced	[130,13		[130]	[133]		
iNKT/T cells	reduced	[131]			[131]		
CD4^{-}iNKT graft	protective	[132]		[132]	[132]		
CECs	reduced	[139,14			[139,140]	[139,14	
mRNAs and EVs							
miR146a	reduced	[155,23		[234]	[155]		
miR155	increased	[153,15		[153]	[155]		
miR181a	reduced	[148]		[148,234]			
miR423, miR199a-	increased	[156,23		[156,157,2	[156,157,2		
miR26b, miR374a, miR489, miR28-5p,	increased	[159]		[159]			
EVs membrane antigens (CD146,	increased	[173]			[173]		
Cytokines and Chemokines							
sIL-2Rα	increased	[188]			[188]		
sST2	increased	[219,22			[219]	[220]	
sST2, CXCL9, OPN,	increased		[203]	[203]	[203]		
CCR5Δ32 allele	protective	[196,19			[196,197]		
CXCL9	Increased		[202,20	[202,203]			
CXCL10. CXCL11	increased		[204]	[204]			
sBAFF	increased		[208]	[208]		[208]	
IL-15	reduced		[209]		[209]		
Proteomics							
IL-2Rα, HGF, IL-8,	increased	[213,21		[213]	[218]	[218]	NCT0022487
REG3α, elafin,	increased	[214,21		[214]	[214,217,2	[217,21	NCT0022487
REG3α, ST2, TNFR1,	increased	[221,22			[221,222,2	[221,22	NCT0280694

aGVHD_MS17	variable	[228]	[229]	[228,229]	[228]		

Table 1. 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

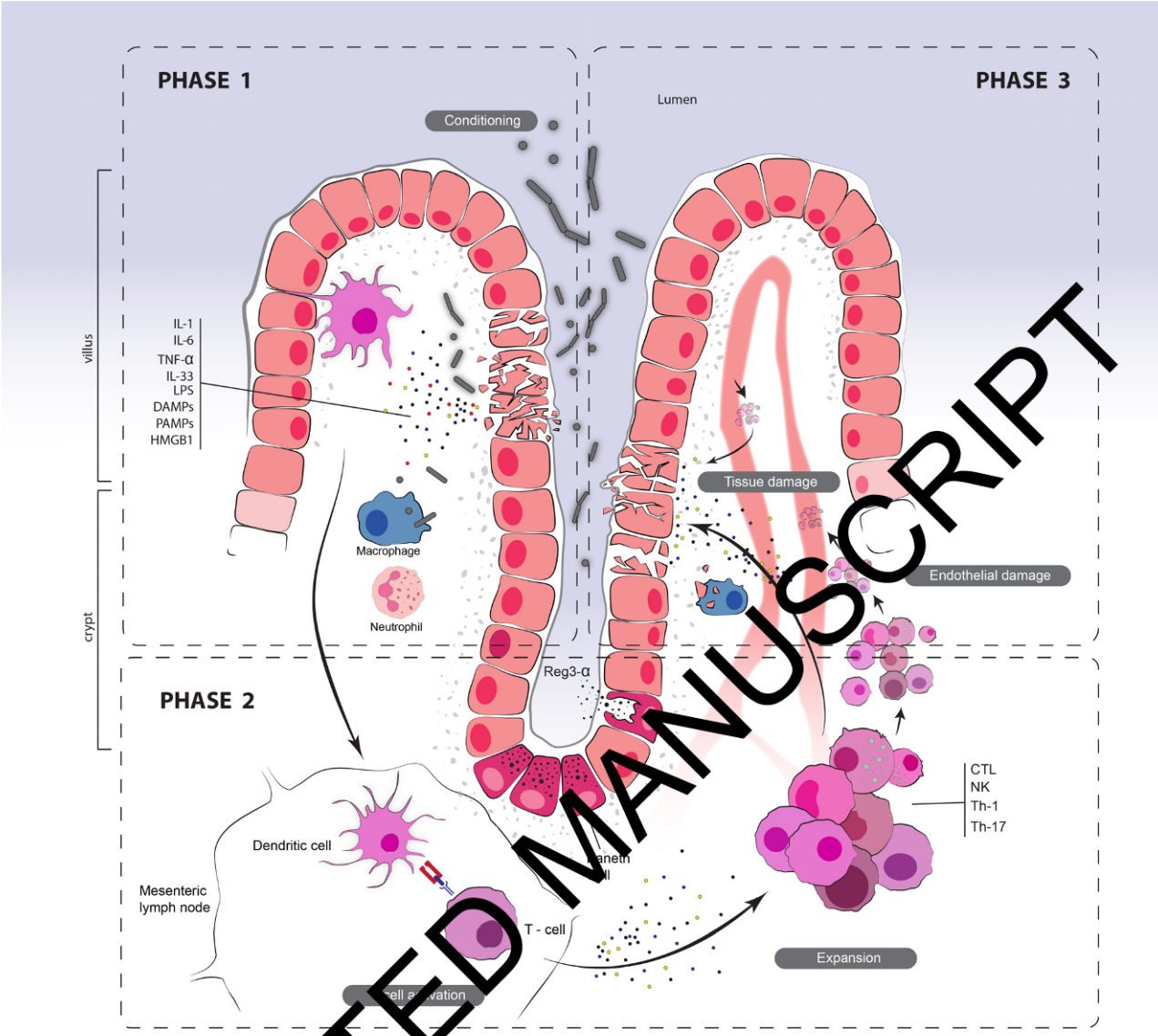

Figure 1 legend

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.