Journal of Clinical Laboratory Analysis

WILEY

REVIEW ARTICLE OPEN ACCESS

Biomarkers of Endothelial Damage in Acute Graft-Versus-Host Disease After Allogenic Hematopoietic Stem Cell Transplantation: Methodological Challenges

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Received: 30 March 2026 | **Revised:** 13 May 2026 | **Accepted:** 10 June 2026

Keywords: biomarkers | circulating endothelial cells | endothelial damage | extracellular vesicles | graft versus host disease | microRNA

ABSTRACT

Background: Acute graft-versus-host disease (aGVHD) is a major complication following allogeneic hematopoietic stem cell transplantation. The current diagnostic approach relies primarily on clinical manifestations and histopathological evaluation of affected tissues. Consequently, there is an urgent need to identify minimally invasive biomarkers that provide predictive, diagnostic, or prognostic value, as well as utility in monitoring therapeutic responses. In particular, biomarkers that enable early identification of patients unresponsive to treatment are highly warranted.

Methods: This article reviews research on aGVHD biomarkers, with a particular focus on those related to endothelial damage. Data published between 2004 and 2025 were sourced from PubMed, Google Scholar, and ScienceDirect using the following keywords: acute graft versus host disease, endothelial damage, biomarkers, circulating endothelial cells, endothelial progenitor cells, microRNA, extracellular vesicles, serum biomarkers, enzyme-linked immunosorbent assay, flow cytometry, and analytical errors. This article reviews the findings, with a specific focus on the analytical challenges associated with their determination and the potential for their implementation in clinical settings.

Results: Recent advances in high-throughput methodologies have led to the identification of potential biomarkers for aGVHD. Growing evidence indicates that endothelial damage contributes to this complication, especially in treatment-resistant patients. In addition to soluble serum biomarkers, other promising candidates such as circulating endothelial cells, extracellular vesicles, and microRNAs have been identified. However, the methodologies for their assessment have not yet been standardized.

Conclusions: Future efforts should focus on standardizing these methods and establishing comprehensive biomarker panels, as none of the biomarkers identified so far are entirely specific to aGVHD.

1 | Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment for many hematological malignancies, particularly in patients with an adverse prognosis. Despite its effectiveness, allo-HSCT is associated with significant post-transplant complications [1]. One such complication is acute graft-versus-host disease (aGVHD), which typically occurs within 100 days after transplantation. Clinical presentations vary from mild to life-threatening [2]. aGVHD remains the leading cause of morbidity and mortality following allo-HSCT, as donor-derived immune cells recognize recipient tissues as foreign, eliciting inflammatory responses and resulting in tissue injury [3]. The initiation phase of aGVHD is associated with host tissue damage, as the preparation of patients for allo-HSCT involves a conditioning regimen that includes high-dose chemotherapy and/or total body irradiation. The primary objectives of conditioning are to eradicate malignant

cells, prepare the bone marrow for stem cell engraftment, and establish immune tolerance in the recipient against the graft [1]. However, the conditioning regimen also causes tissue damage, leading to the exposure of damage-associated molecular patterns (DAMPs), the production of proinflammatory cytokines, and the infiltration of immune cells [4]. This process activates antigen-presenting cells (APCs). Additionally, damage to the intestinal wall increases the translocation of bacterial components and metabolites through the compromised intestinal barrier. Exposure to pathogen-associated molecular patterns (PAMPs), along with alterations in the gut microbiota due to conditioning and antibiotic treatment, further contributes to APC activation [4, 5]. Activated APCs subsequently activate donor immune cells, primarily T cells [6]. A major risk factor for the development of aGVHD is the disparity in human leukocyte antigens (HLA) between the stem cell donor and recipient [7]. In the final stage, these activated "alloreactive" T cells migrate into target tissues, causing tissue damage, which is also associated with the release of additional proinflammatory cytokines, leading to progressive inflammation and tissue injury [8]. Commonly affected organs include the gastrointestinal tract, liver, and skin; however, atypical presentations may involve other organs (Figure 1) [3]. While endothelial cells play a crucial role in the migration of immune cells from the circulation to tissues, they can also function as non-classical APCs and may be initial targets of alloreactive T cells [9, 10].

Endothelial damage is frequently observed in histopathological assessments of aGVHD-affected tissue. Endothelial injury also persists in steroid-refractory GVHD (SR-GVHD), which is associated with high mortality [11]. In addition to alloreactive lymphocytes, predominantly during aGVHD, many other factors contribute to endothelial damage, including conditioning regimens and infections [12]. Sustained endothelial activation and injury may also provoke additional complications, such as transplant-associated thrombotic microangiopathy (TA-TMA), a highly lethal complication of allo-HSCT [13].

The diagnosis of aGVHD currently relies mainly on clinical manifestations and histopathological assessment of affected tissues [3]. However, initial clinical manifestations do not necessarily correlate with long-term survival outcomes; as the disease progresses, persistent inflammation often makes subsequent immunosuppression ineffective. In contrast, for treatment

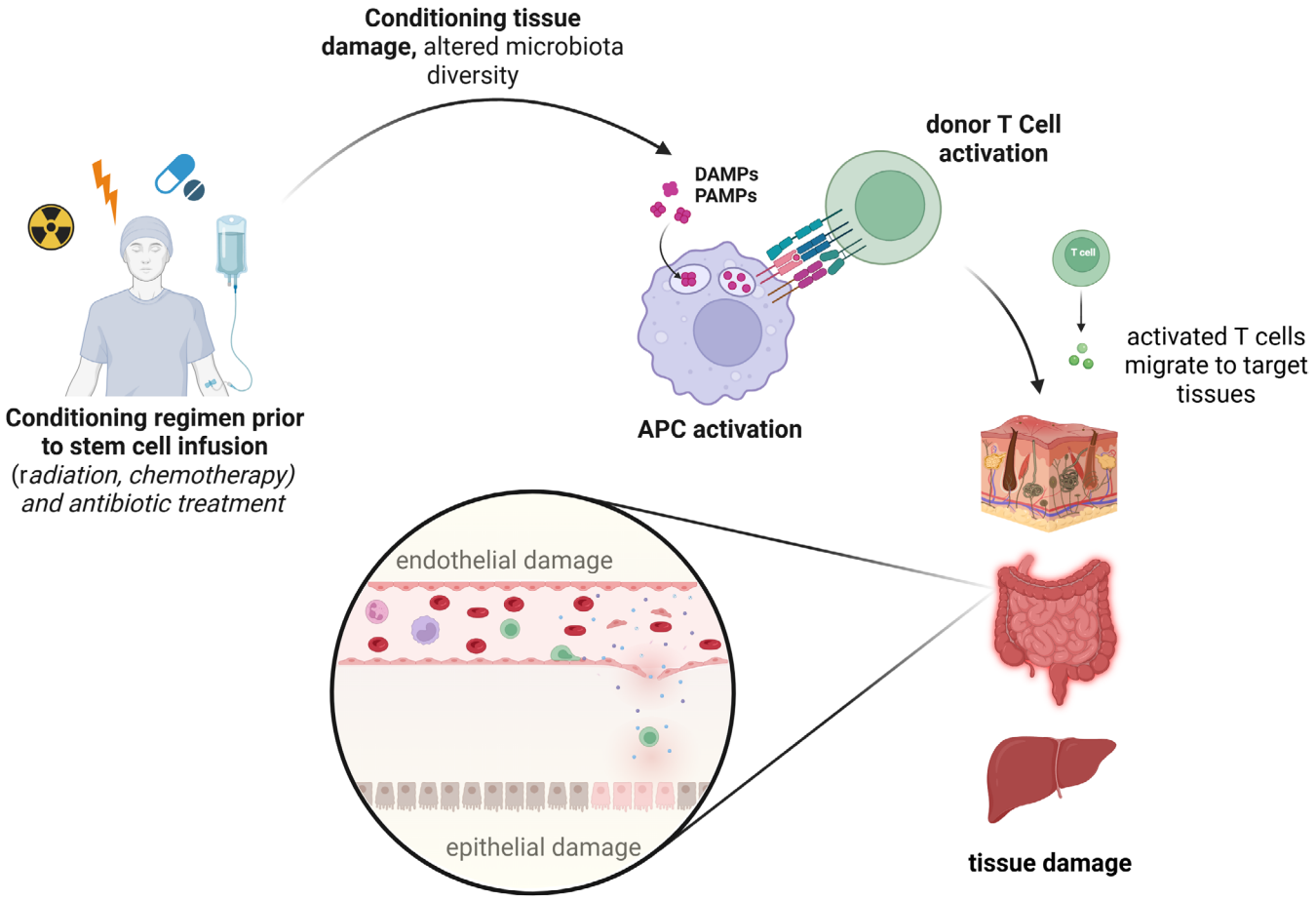

FIGURE 1 | The conditioning regimen induces tissue damage, resulting in the exposure of damage-associated molecular patterns (DAMPs) and the production of proinflammatory cytokines. This process also facilitates the translocation of bacterial components and metabolites, known as pathogen-associated molecular patterns (PAMPs), through the compromised intestinal wall. Consequently, antigen-presenting cells (APCs) are activated. These activated APCs, in turn, stimulate donor T cells. In the final stage, the activated "alloreactive" T cells migrate into target tissues, causing further tissue damage, which is associated with the release of additional proinflammatory cytokines, leading to progressive inflammation and tissue damage. Commonly affected organs include the gastrointestinal tract, liver, and skin. In addition to epithelial damage, endothelial damage is also present in aGVHD. Created with https://biorender.com.

responders, excessive immunosuppression can increase the risk of infections and reduce the graft-versus-leukemia effect, raising the likelihood of disease relapse [14]. Consequently, minimally invasive blood-based biomarkers that enable early prediction, diagnosis, prognosis, and therapeutic monitoring are highly warranted. However, methodological challenges for biomarker quantification should be taken into account in the future. A summary of promising biomarkers of aGVHD, along with commonly used methods for their quantification and their limitations, is present in Table 1.

2 | Serum Biomarkers

The most investigated biomarkers in the field of aGVHD are soluble serum or plasma markers, primarily substances released into the bloodstream from activated (immune) cells and/or damaged tissue involved in the pathophysiology of aGVHD [50]. There is a growing body of evidence linking aGVHD to biomarkers of endothelial damage and vulnerability [12, 50]. One of them is suppression of tumorigenicity 2 (ST2), which is an interleukin-33 (IL-33) receptor. Two isoforms of ST2 are known: a transmembrane receptor expressed mainly on T cell subtypes and soluble ST2 (sST2), which acts as an IL-33 decoy receptor and is secreted by injured or activated endothelial or epithelial cells and fibroblasts [51, 52]. High sST2 levels are associated with increased non-relapse mortality (NRM), SR-GVHD, and TA-TMA [52, 53].

Angiopoietin 2 (ANG2), a growth factor involved in endothelial cell inflammatory responses and angiogenesis, is another potential indicator of endothelial vulnerability [54, 55]. Elevated pre-transplant concentrations of ANG2 are correlated with increased NRM and SR-GVHD, but this association has been observed only in patients who subsequently develop high-grade aGVHD [15]. Furthermore, elevated post-transplant levels of ANG2 are also associated with SR-GVHD and TA-TMA [13, 15]. This finding aligns with the "three-hit hypothesis," which suggests that three sequential events are necessary to initiate TA-TMA. The first event involves a predisposition to complement activation or pre-existing endothelial vulnerability. The second event is toxicity induced by conditioning regimens. The third event, which triggers TA-TMA, involves additional endothelial injury due to infections, prophylactic immunosuppression, or alloreactivity in aGVHD [56].

To date, none of the identified potential biomarkers are completely specific for aGVHD. Therefore, some research groups have focused on determining biomarker panels for aGVHD diagnosis, prognosis, and therapeutic monitoring. One of them is the Ann Arbor panel, which categorizes patients at the onset of aGVHD based on the risk of treatment failure and NRM. This panel involves measuring regenerating family member 3 alpha (REG3α), sST2, and tumor necrosis factor receptor 1 (TNFR1) [14]. With the development of more sensitive tests for sST2, the Mount Sinai Acute GVHD International Consortium (MAGIC) developed the MAGIC Algorithm Probability (MAP) in a multicenter study, which includes serum concentrations of REG3α and sST2. MAP before aGVHD onset (day 7), at onset, and 1 week after initiation of systemic treatment predicts long-term outcomes. MAP determined at the onset of GVHD allows stratification of patients into three groups (low, medium, and high risk), while MAP before aGVHD onset and after initiation of systemic treatment stratifies patients into two groups (low and high risk) [16, 17]. It was also demonstrated that MAP prior to the initial administration of Ruxolitinib, a second-line treatment for aGVHD, can predict treatment response and NRM. However, patients with elevated MAP exhibit poor survival irrespective of the treatment administered, whereas patients with low MAP who received ruxolitinib had better survival compared to those who did not [18]. In addition, sST2 and TNFR1 levels were also increased before treatment with Ruxolitinib in non-responders compared to responders [19].

Elevated levels of sST2 are associated with tissue damage, including endothelial damage [57]. In contrast, increased levels of TNFR1 serve as an inflammatory marker, as TNFR1 is the receptor for tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine involved in the pathogenesis of aGVHD. TNFR1 is considered a surrogate and more stable marker for TNF-α [58]. REG3α serves as a specific biomarker for gastrointestinal aGVHD (GI-GVHD), which is associated with higher NRM than other forms of aGVHD [20, 57]. REG3α is a lectin secreted by Paneth cells into the intestinal lumen. During intestinal injury, its concentration in peripheral blood increases [57].

In addition to the aforementioned serum biomarkers of endothelial injury, a standardized biomarker panel called the Endothelial Activation and Stress Index (EASIX) has been developed as a surrogate marker for endothelial injury. This index is calculated from routinely measured laboratory parameters, including serum lactate dehydrogenase (LDH) concentrations, creatinine concentrations, and platelet counts [23]. Elevated EASIX levels in patients undergoing allo-HSCT, assessed at various time points before and after transplantation, have been associated with increased NRM and decreased overall survival (OS) [24, 25].

In addition to its association with OS and TRM, elevated EASIX prior to conditioning (EASIX-pre) has been linked to a higher risk of TA-TMA [26, 27]. Parallel dynamics in TNFR1 and log2-EASIX levels have also been observed after transplantation, with both markers significantly elevated in patients who developed high-grade aGVHD. An increased log2-EASIX at day 7 has been identified as a predictor for the development of grade II-IV aGVHD, as well as for a higher risk of NRM and reduced OS [25]. Furthermore, EASIX measured at the onset of aGVHD (EASIX-GVHD) has been shown to predict survival outcomes in patients undergoing reduced-intensity conditioning [23]. Its predictive value at day 0 has also been demonstrated for sinusoidal obstructive syndrome, another endothelial complication following allo-HSCT [59]. Additionally, the utility of EASIX in predicting survival across various hematological and infectious conditions has been established [60–62]. It has also been recognized as a predictor of severe endothelial complications following chimeric antigen receptor T cell therapy [63].

Recent advancements in high-throughput proteomics methodologies have enabled the identification of numerous potential biomarkers of aGVHD. However, in validation research, the most commonly used method is the enzyme-linked immunosorbent assay (ELISA), which can also be applied in clinical

TABLE 1 | Overview of promising biomarkers of aGVHD, their possible clinical significance, commonly used analytical methods for their quantification and their advantages and limitations for clinical use.

Biomarkers	Possible clinical significance	Commonly used analytical method	Advantages	Limitations	Potential solutions to the limitations	References
Serum biomarkers (sST2, REG3α, ANG2, TNFR1, MAP)	Predictive, prognostic, diagnostic, treatment monitoring	ELISA	Efficiency, accessibility	Inconsistencies across results because of differences between antibodies and calibrators used	Use of antibodies with established specificity and sensitivity, development of international reference materials, establishment of external quality assessment programs	[13–22]
EASIX (LDH, creatinine, platelets)	Predictive, prognostic	Standardized biomarker panel composed of standard laboratory parameters	Composed of standard laboratory parameters, easily accessible, rapid, cost-effective	An indirect marker of endothelial damage, possible influence of confounding factors	Interpretation of results with consideration of influencing factors	[23–28]
Circulating endothelial cells	Predictive, differential diagnostic, treatment monitoring	Flow cytometry	Direct marker of endothelial damage, simultaneous identification of other cell populations	Sample stability, lack of consensus on immunophenotype and standardized methodology	Consensus on immunophenotype	[29–32]
Extracellular vesicles	Predictive, prognostic, differential diagnostic, treatment monitoring	Flow cytometry	Found in various biological samples, composition depends on parental cell (status)	Small size and low antigen expression, recommendations for management of (pre)analytical factors are needed	Comprehensive reporting to support preparation of recommendations for management of (pre)analytical factors, calibration of flow rate, fluorescence, and light scatter	[33–42]
miRNA	Predictive, prognostic, diagnostic	RT-qPCR	Found in various biological samples, more stable than mRNA	Recommendations for management of (pre)analytical factors are needed	Comprehensive reporting to support preparation of recommendations for management of (pre) analytical factors, determination of appropriate normalization strategy, and reference control	[34, 43–49]

Abbreviations: ANG2, angiopoietin 2; EASIX, endothelial activation and stress index; ELISA, enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase; MAP, mount sinai acute GVHD international consortium algorithm probability; REG3α, regenerating family member 3 alpha; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; sST2, soluble suppression of tumorigenicity 2; TNFR1, tumor necrosis factor receptor.

settings [21]. To ensure reliable results, it is essential to validate ELISA kits. This process typically involves assessing linearity, determining precision (reproducibility), evaluating the assay's dynamic range, and conducting recovery experiments to identify potential interferences in the sample. It is also advisable to establish positive and negative controls on each plate as part of daily quality control, analyze samples in duplicate, and confirm the goodness-of-fit of the calibration curve. Because the calibration curve cannot be extrapolated, it is necessary to prepare adequately diluted samples [64]. However, differences between antibodies and calibrators used in assays from different manufacturers can result in inconsistencies across results, making direct comparisons difficult [21, 22]. On the other hand, EASIX, which is composed of standard laboratory parameters, is easily accessible, rapid, and more cost-effective. Nonetheless, EASIX is an indirect marker of endothelial damage and may be confounded by factors such as conditioning intensity, platelet transfusions, or other causes of changes in platelet count, LDH, or creatinine, regardless of endothelial damage [23, 28].

3 | Circulating Endothelial Cells and Endothelial Progenitor Cells

Circulating endothelial cells (CECs) are endothelial cells released into the bloodstream in response to vascular injury, serving as potential biomarkers of vascular damage [65]. These cells have been studied in various diseases, including aGVHD [29, 66–69]. Almici et al. observed an increase in CECs from the time of engraftment, with peaks at the onset of aGVHD. A decrease in CECs was also noted following a response to treatment [29, 30]. Due to the rapid kinetics of CECs, measuring at the disease onset was recommended to help differentiate from other complications that do not involve endothelial damage. At engraftment, significantly higher CEC counts were identified in patients without aGVHD [29, 30]. Similarly, Takamatsu et al. reported significantly lower CEC levels 7 days post-transplantation in patients who later developed aGVHD [31]. However, these findings should be validated in larger cohorts and interpreted with consideration of potential influencing factors such as conditioning regimens, other endothelial dysfunction syndromes, infections, and the effects of medications used [29, 70–72].

Besides CECs, endothelial progenitor cells (EPCs) can also be present in peripheral blood [73]. Both cell populations have the potential to serve as biomarkers for assessing endothelial dysfunction [74]. Given the evidence of endothelial damage and increased angiogenesis in organs affected by aGVHD, EPCs have been investigated in this context [29, 75], because of their vascular regenerative potential, involvement in angiogenesis, and possible immunomodulatory effects [76]. However, findings across studies have been inconsistent. Some animal model studies have shown that the depletion of EPCs can alleviate aGVHD [77]. Additionally, elevated levels of EPCs, characterized as CD34+/CD133+/CD309+, have been detected in the peripheral blood of patients with aGVHD, and these levels remain elevated in cases of SR-GVHD [78]. Conversely, other studies have not observed an increase in EPCs after day 14 in patients with aGVHD, unlike in those without this complication [31]. Furthermore, research using animal models has indicated that infusion of bone marrow-derived EPCs, defined as CD45-/CD31+/VEGFR2+, can ameliorate aGVHD. This is accompanied by a reduction in T cell infiltration and integration of EPCs into damaged endothelium [79]. Moreover, a lower concentration and impairment of bone marrow EPCs, identified as CD34+/VEGFR2+/CD133+, have been associated with the severity of aGVHD and aGVHD-related cytopenia [80].

One potential reason for inconsistencies in results is the lack of consensus regarding the immunophenotype and the absence of standardized methodology. The CellSearch system, used by Almici et al. for CEC quantification, is a commercially available, standardized platform for rare cell immunomagnetic isolation and quantification and therefore holds potential for clinical use. Another limiting factor is the poor stability of samples; thus, samples must be analyzed as soon as possible. However, for analysis with CellSearch, samples can be drawn into preservative tubes, which stabilize them and allow analysis up to 96 h after venipuncture. CellSearch allows determination of only three different fluorochromes simultaneously, whereas flow cytometry, which is the most commonly used method in the literature, enables simultaneous detection of more cell markers. This can increase the specificity of CEC determination and allows for the simultaneous identification of other cell populations, such as EPCs. Both methods have potential for clinical application, and their comparability has been demonstrated. However, the unreliability of leukocyte counts in peripheral blood during bone marrow aplasia can limit the double-counting approach. Determination of leucocyte count using counting beads can reduce this limitation [32]. Flow cytometers are usually available in allo-HSCT centers because of their role in diagnosis and treatment monitoring of various hematological diseases. However, due to the lack of consensus on CEC and especially EPC immunophenotypes, as well as the lack of standardized methodology, it is recommended that each laboratory determine its own cutoff values and monitor dynamic changes in these cells [81, 82]. In addition, further studies on EPC (subtypes) immunophenotype determination and their significance in aGVHD are warranted [76, 81]. Elevated CEC counts after allo-HSCT indicate endothelial damage during transplantation [29]. However, CECs and EPCs are rare cell populations in peripheral blood; therefore, a reliable analysis requires an adequate number of cells. For flow cytometric analysis, it is recommended to add markers for cell viability and nuclear identification, as well as to use isotype controls to exclude nonspecific events. Moreover, establishing a dump channel is advised to exclude cells not of interest, such as CD45 to exclude leukocytes [81, 83, 84].

4 | Extracellular Vesicles

Possible biomarkers of vascular injury and increased thrombotic risk also include extracellular vesicles (EVs) [85]. These membrane-enclosed particles are released from cells and are involved in intracellular communication. Their composition depends on the type, (patho)physiological state, and activation state of the parental cell, and their release mechanism. Since they can be found in various body fluids, they represent a promising biomarker in different diseases, including aGVHD [86].

Lia et al. demonstrated that in patients with multiple myeloma undergoing allo-HSCT, increased CD146 fluorescence on EVs

was related to a higher risk of aGVHD, whereas CD31 fluorescence and CD140α concentration were linked to a reduced risk of aGVHD. Significant changes in the signal levels of these markers were observed prior to the onset of aGVHD. Notably, CD146 is expressed not only on endothelial cells, but also on a subset of T cells. Given the correlation between CD146 and other endothelial markers, the authors suppose that CD146+ EVs are primarily of endothelial origin. However, these observations require further validation in future studies. CD31 is also expressed on endothelial cells, as well as on T cells and APCs, potentially playing a role in preventing lymphocyte excessive responsiveness [33]. These findings were subsequently supported by the same research group in patients following haploidentical allo-HSCT. Additionally, the combination of TNFR1 concentration, CD146 fluorescence on EVs, and miR-100 or miR-194 expression in EVs was identified as the most effective in distinguishing patients with and without aGVHD at disease onset [34]. In earlier studies, elevation of endothelial EVs, identified as CD62E+ EVs, was observed in patients with aGVHD [35, 36]. Higher levels of endothelial EVs were also observed several years after transplantation in allo-HSCT survivors who had undergone myeloablative conditioning. Furthermore, patients with a history of TA-TMA exhibited higher levels of erythrocyte EVs (EryEVs) [85]. In another study of patients with aGVHD, an elevation in EryEVs was observed at disease onset, which was not detected in patients post-allo-HSCT with infectious complications [37]. Similarly, elevated levels of EryEVs and total EV count, based on annexin V positivity at engraftment, were connected with an increased risk for aGVHD, with the connection being stronger in patients undergoing reduced-intensity conditioning [38]. Regarding EV determination in aGVHD, lymphocyte EVs were also studied; an increase in CD3+CD8+ EVs, and particularly CD3+HLA-DR+ EVs, were associated with aGVHD severity, while changes following treatment initiation indicated responsiveness to therapy. No increase was observed in engraftment syndrome or viral reactivation [39]. Additionally, pre-transplant elevated serum concentrations of CD69+ EVs, presumably derived from resident T cells, represent a potential predictive biomarker for aGVHD [40].

Recent studies indicate that EVs have potential as predictive, diagnostic, prognostic, and responsive biomarkers in aGVHD. However, their precise roles in the pathophysiology of aGVHD require further investigation. Notably, these studies have revealed numerous preanalytical and analytical discrepancies. Flow cytometry is a commonly used method for EV analysis and shows promise for application in clinical laboratories. The basic principle of EV flow cytometry analysis is schematically illustrated in Figure 2. Nevertheless, methodological challenges in EV determination must be addressed before their potential clinical implementation. The analysis of EVs is challenging because of their small size and significantly dimer antigen expression on their surface compared to cells, resulting in low light scatter and fluorescence signals. Therefore, highly sensitive flow cytometers are needed for their detection. However, the sensitivity and detection ranges of flow cytometers vary, complicating direct comparisons of results across different analyzers, particularly due to the heterogeneity in EV size and composition [41, 42, 86]. Consequently, calibration of flow rate, light scattering, and fluorescence intensity using traceable reference materials is essential to ensure reproducibility [87]. Furthermore, the most frequently used materials for light scattering calibration are polystyrene or silica beads, which have a higher refractive index than EVs. Therefore, a model based on Mie theory was developed to correlate the light scattering properties of EVs with their size. This model assumes that EVs are spherical particles and employs a refractive index assumption derived from literature data on biological particles with a comparable structure [88].

Numerous particles of similar size to EVs are present in blood, potentially interfering with their analysis. Consequently, isolation is sometimes necessary prior to the analysis. However, due to the heterogeneity of the EV population and the overlap in properties between EVs and non-EV particles, no isolation method is perfect. While flow cytometry allows analysis in plasma, serum, or even whole blood, isolation may still be required to detect rare EV populations, with the awareness that some EV subpopulations may be excluded from analysis [42, 89]. In the aforementioned studies, various isolation methods were used, many of which are labor-intensive or time-consuming and thus unsuitable for routine clinical practice. This is further complicated by the need for equipment not commonly available in clinical laboratories, making clinical translation more difficult. Another limitation of EV analysis is the lack of a reagent that can specifically recognize all EV types. Additionally, determining the origin of EVs is challenging, as most markers are expressed across diverse cell populations, making it difficult to ascertain the parental cell origin based solely on a single marker, except for CD235a, which is the most commonly used marker for erythrocyte EV identification. Therefore, identifying cell populations typically requires a combination of various markers. In EV determination, their heterogeneity must be considered, and the number of markers that can be used within a single panel is limited because of potential steric hindrance from their small size. Moreover, the brightness of the fluorophores can affect the results. Therefore, the use of bright fluorophores without spectral overlap is recommended [42, 90–92].

Because measurements are performed near the detection limit and possible interferences such as protein complexes or lipoproteins may be present, numerous controls must be analyzed to ensure reliable results. These controls include buffer-only control, buffer with reagent control, unstained control, isotype control, fluorescence minus one control, single-stained control, detergent treatment control, and procedural control. Additionally, serial dilution is crucial to prevent swarm detection, which occurs when hundreds of particles cross the detector simultaneously [42, 93].

Given the numerous analytical and preanalytical factors that can influence results, comprehensive reporting is essential. Consequently, various working groups have developed frameworks outlining the minimal information required for reporting on the analytical and preanalytical phases (e.g., Minimal Information for Studies of Extracellular Vesicles (MISEV), MIBlood-EV and a framework for standardized reporting of EV flow cytometry experiments (MIFlowCyt-EV)). These frameworks aim to facilitate comparison between studies and enable future meta-analyses to establish detailed recommendations, which are critical prior to clinical implementation [94–96]. Nevertheless, research on EVs in aGVHD, in addition to methodological differences, often lacks detailed reporting and calibration, complicating direct comparative analyses and reproducibility.

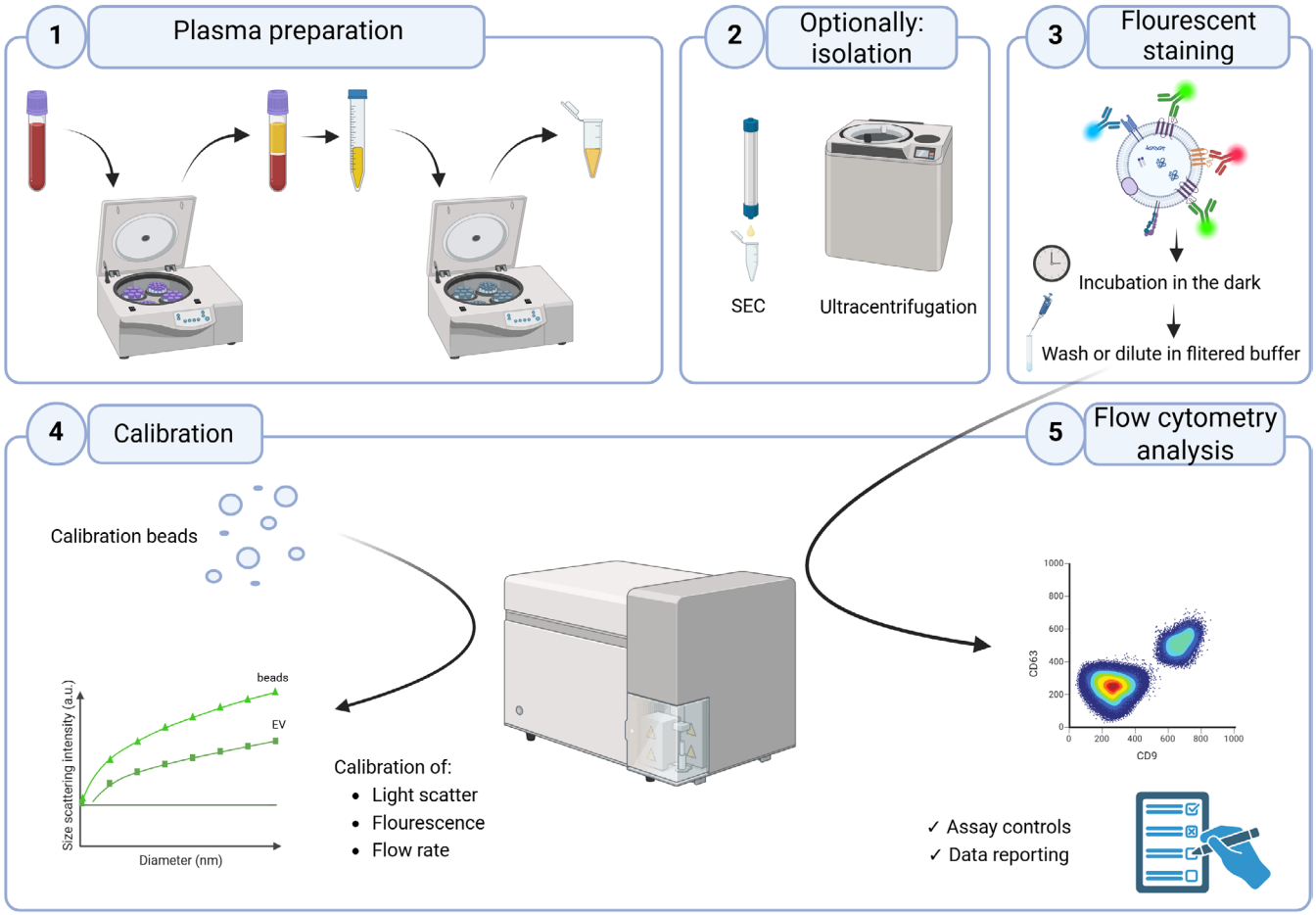

FIGURE 2 | Schematic representation of extracellular vesicle (EV) flow cytometry analysis (1) After blood collection, the sample is centrifuged. The most commonly used protocol is double centrifugation: the first spin removes cells, and the second centrifugation of the transferred plasma eliminates residual platelets and debris. (2) Optionally, isolation procedures such as size exclusion chromatography (SEC) or ultracentrifugation may be used to enrich EVs and reduce background contaminants. (3) EVs are stained with fluorescent antibodies and/or dyes to specifically label EV markers. After incubation, the stained samples are washed or diluted with filtered buffer. (4) Before sample analysis, flow cytometers must be calibrated with reference beads to convert arbitrary units into standardized units. Calibration of light scatter, fluorescence, and flow rate is recommended. (5) After sample acquisition with the flow cytometer, the data are analyzed and interpreted in conjunction with analysis of controls. To ensure reproducibility, it is essential to report (pre)analytical factors. Created with https://BioRender.com.

5 | Micro RNA

As previously mentioned, micro RNAs (miRNAs) also have the potential to serve as biomarkers of aGVHD. These are small non-coding RNAs that play a pivotal role in the post-transcriptional regulation of gene expression [97].

Many different miRNAs have been identified as differentially expressed in patients with and without aGVHD and therefore have potential as predictive, diagnostic, and prognostic biomarkers [34, 43–45]. Among them, miR-155 is one of the most studied in this field. Upregulation of miR-155 in EVs has been demonstrated in the peripheral blood of patients with aGVHD even before the onset of this complication. In addition, in vitro studies have shown that miR-155 transported in endothelial EVs is involved in T cell differentiation and thus may contribute to aGVHD initiation, making it a possible drug target [46]. Additionally, miR-100 has been observed to be involved in the regulation of neovascularization in inflamed tissue during aGVHD [47]. Nonetheless, the majority of differentially expressed miRNAs in patients with aGVHD are involved in the regulation of the immune response [34, 43, 44].

miRNAs can be quantified in various biological samples. In peripheral blood, they are transported in EVs or attached to serum proteins which makes them relatively stable compared to mRNAs [48]. However, there are other preanalytical and analytical challenges that need to be addressed before miRNA analysis can be implemented in clinical practice. These include sample stability, isolation efficacy, and matrix-specific variability [49]. In some studies, miRNAs were quantified in whole blood [98], capturing both cellular and extracellular fractions, while others focused on total cell-free miRNAs from serum or plasma [43, 44, 99]. miRNAs isolated from EVs were also analyzed [34, 43]. In some cases, differences between cell-free and EVs associated miRNAs were observed [46]. However, the extent to which these differences are caused by the choice of EVs isolation method should be further assessed. Methodological

differences can also affect the results of miRNA expression analysis. The most accurate method for their quantification is reverse transcription-quantitative polymerase chain reaction (RT-qPCR) which should be used to confirm results from high-throughput methods such as miRNA sequencing or microarrays. Since many steps are involved in the analysis, ranging from miRNA isolation to ligation, reverse transcription, and amplification, the addition of spike-in controls is recommended to monitor workflow efficiency. One of the major challenges in miRNA analysis is the data normalization strategy. Therefore, it is advisable to determine a reference control for each sample type and study population [45, 49, 100–102].

6 | Conclusion

Despite significant advancements in allo-HSCT in recent years, aGVHD remains a substantial and potentially life-threatening complication. The diagnosis of aGVHD relies primarily on clinical presentation, which is often non-specific. Additionally, at disease onset, clinical manifestations do not necessarily correlate with patient outcomes. Therefore, blood-derived biomarkers could be advantageous, particularly those that help identify patients who are resistant to treatment or more susceptible to developing additional complications such as TA-TMA. Numerous studies have highlighted the role of endothelial injury in aGVHD. Thus, early detection of endothelial vulnerability and damage may guide risk-stratified prophylaxis in allo-HSCT recipients. However, endothelial injury can also be associated with some other post-transplant complications, including engraftment syndrome, TA-TMA, sinusoidal obstructive syndrome, and infections. The main challenge in applying biomarkers is therefore the variability of diagnostic and inclusion criteria across studies, along with the lack of standardized methods for biomarker quantification. Future research should prioritize standardizing preanalytical and analytical factors and ensuring precise clinical interpretation of results, which requires collaboration between clinicians and laboratory specialists. Furthermore, endothelial injury is not exclusive to aGVHD, and none of the biomarkers identified so far are specific to aGVHD. Therefore, developing distinct biomarker panels is expected to be highly advantageous in the future.

Author Contributions

Conception and design of the work, K.K., H.P.; acquisition and interpretation of data, K.K., H.P., writing – original draft preparation, K.K., H.P.; writing – review and editing, K.K., H.P.; final approval of the version to be published, K.K., H.P., all aouthors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

This work was supported by UMC LJubljana, TP 20240011.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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