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# Circulating miRNA panel for prediction of acute graft-versus-host disease in lymphoma patients undergoing matched unrelated hematopoietic stem cell transplantation

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**Acute graft-versus-host disease (aGVHD) results in significant morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Noninvasive diagnostic and prognostic tests for aGVHD are currently lacking, but would be beneficial in predicting aGVHD and improving the safety of allo-HSCT. Circulating microRNAs exhibit marked stability and may serve as biomarkers in several clinical settings. Here, we evaluated the use of circulating microRNAs as predictive biomarkers of aGVHD in lymphoma patients after allo-HSCT from matched unrelated donors (MUDs). After receiving informed consent, we prospectively collected plasma samples from 24 lymphoma patients before and after unmanipulated MUD allo-HSCT; microRNAs were then isolated. Fourteen patients developed aGVHD symptoms at a median of 48 days (range: 32–90) post-transplantation. Two patients developed intestinal GVHD, eight cutaneous GVHD, and four multiorgan GVHD. The microRNA expression profile was examined using quantitative real-time polymerase chain reaction (qRT-PCR). MicroRNAs 194 and 518f were significantly upregulated in aGVHD samples compared with samples taken from non-aGVHD patients. Remarkably, these upregulated microRNAs could be detected before the onset of aGVHD. Pathway prediction analysis indicated that these microRNAs may regulate critical pathways involved in aGVHD pathogenesis. Considering the noninvasive characteristics of plasma sampling and the feasibility of detecting miRNAs after allo-HSCT using real-time polymerase chain reaction, our results indicate that circulating microRNAs have the potential to enable an earlier aGVHD diagnosis and might assist in individualizing therapeutic strategies after MUD allo-HSCT. Nevertheless, standardization of blood sampling and analysis protocols is mandatory for the introduction of miRNA profiling into routine clinical use.** 

Graft-versus-host disease (GVHD) represents the leading cause of non-relapse mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. Several risk factors for the development of GVHD have been identified, such as the degree of donor–recipient match at the human leukocyte antigen (HLA) loci, the type of conditioning regimen, the stem cell source, and gender disparity [2]. However, moderate to severe acute GVHD (aGVHD) still occurs in up to 40% of patients receiving HLA-identical grafts, and the incidence is higher among recipients of matched unrelated donor grafts [3]. Limited progress has been made in the treatment of aGVHD, and the outcome for patients with steroid-refractory aGVHD is poor, with a mortality rate approaching 70% and no therapy available to improve survival [4]. For these reasons, aGVHD prevention through improved prophylaxis strategies has been a primary focus of patient management after allo-HSCT. This highlights the importance of identifying predictive markers of aGVHD in its early stages to decrease

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aGVHD-related morbidity and mortality and to provide clinicians with a potential time window to abort the alloreactive process at its beginning.

So far, no validated diagnostic blood test has proven able to predict the onset of aGVHD. It has been reported that increased concentrations of tumor necrosis factor (TNF) α during allo-HSCT conditioning are predictive of aGVHD [5], and elevated post-HSCT/pre-HSCT TNFα ratios at day 7 are predictive of aGVHD [6–8] in the myeloablative setting. Furthermore, increased concentrations of interleukin (IL)-6 during the first 3 weeks after HSCT predicted later onset of aGVHD [9]. Altered levels of the cytotoxic T-cell proteins CD40L, CD8, and IL-2 receptor (IL-2R) at day 15 post-HSCT have been associated with subsequent development of aGVHD [10]; however, these findings await confirmation. In addition, increased soluble B cell-activating factor (sBAFF) concentrations in the early post-HSCT period have been associated with a decreased risk of developing aGVHD [11]. Proteomic studies led to the identification of a panel of four informative proteins, namely, tumor necrosis factor receptor 1 (TNFR1), IL-2Rα, IL-8, and hepatocyte growth factor (HGF), whose detection at the onset of aGVHD has diagnostic and prognostic value [12]. These proteins are found at the onset of the disease, but do not predict aGVHD development. The same researchers have discovered GVHD biomarkers, specific to target organs, that allow different forms of aGVHD to be distinguished [13,14].

MicroRNAs (miRNAs) emerged as a novel class of biomarkers with potential clinical relevance. miRNAs are short (22–25 nucleotides), noncoding RNAs that negatively regulate gene expression at the post-transcriptional level by binding to their target messenger RNA (mRNA) in a sequence-specific manner [15,16]. MicroRNAs play an essential role in fundamental biological processes, such as differentiation, proliferation, apoptosis, and homeostasis [17], and are involved in different diseases, including cancer, that exhibit a tissue-specific deregulation pattern [18]. MicroRNAs are shed into the bloodstream in a very stable form and can be detected by miRNA profiling approaches such as quantitative real-time polymerase chain reaction (qRT-PCR) and microarray [19–21]. Given their tissue specificity and their stability in many body fluids, microRNAs have received a great deal of attention with respect to their potential as minimally invasive biomarkers for several conditions [22,23], including aGVHD [24].

Our intent was to perform a prospective observational study in which levels of circulating plasma miRNAs were assessed with a qRT-PCR-based high-throughput miRNA array in lymphoma patients after allo-HSCT from matched unrelated donors (MUDs). We planned to identify noninvasive biomarkers able to predict aGVHD at early stages after allo-HSCT, therefore enhancing therapeutic success and increasing the life expectancy of allografted patients.

## Methods

*Patient characteristics and sample collection*

Twenty-four patients were enrolled in a prospective study aimed at the assesment of circulating miRNA levels in a high-throughput fashion at several points before and after transplantation. Approval was obtained from the institutional review board (Study No. D255690 INT 97/11), and all patients gave their written informed consent. The stem cell source for allo-HSCT was peripheral blood in all cases. The conditioning regimen consisted of a combination of thiotepa (10 mg/kg) and cyclophosphamide (100 mg/kg; $n = 14$), with the addition of fludarabine (120 mg/m$^2$) in 10 cases [25]. The grafts were in vivo depleted of T cells using rabbit antithymocyte globulin (thymoglobulin 3.5 mg/kg/day on days −4 and −3) or alemtuzumab in 23 cases and one case, respectively. Post-transplantation GVHD prophylaxis consisted of cyclosporine A and short-course methotrexate (10 mg/m$^2$ on day +1, 8 mg/m$^2$ on days +3 and +6; $n = 22$) or cyclosporine A and mycophenolate (15 mg/kg three times daily starting from day 0; $n = 2$). The median graft composition was 6.9 × 10$^6$ CD34$^+$ cells/kg (range 3.3–11.4) and 2.99 × 10$^8$ CD3$^+$ cells/kg (range 1.47–5.46). Patient characteristics, conditioning regimens, donor type, and aGVHD characteristics are summarized in Table 1. After giving informed consent, 20 healthy donors were also included in the study as the normal counterpart. To obtain plasma, peripheral blood samples (3 mL) were collected in EDTA (BD Vacutainer, K2 EDTA, Ref. 367856) and centrifuged within 1 hour at 1,200*g* for 10 min at 4°C to spin down the blood cells. The

**Table 1.** Characteristics of patients

| Characteristic | GVHD group ($n = 14$) | Non-GVHD group ($n = 10$) |
|---|---|---|
| Median age | 50 (22–65) | 34 (18–56) |
| Median dose/kg | | |
| CD34$^+$ | 7.8 × 10$^6$ (5.9–11.4) | 6.5 × 10$^6$ (3.3–9) |
| CD3$^+$ | 2.96 × 10$^6$ (1.47–5.46) | 3 × 10$^6$ (1.58–4.48) |
| Disease type | | |
| Non-Hodgkin's lymphoma | 8 (57%) | 4 (40%) |
| Hodgkin's lymphoma | 6 (43%) | 6 (60%) |
| Donor type | | |
| Matched unrelated | 14 (100%) | 10 (100%) |
| Regimen type | | |
| Thiotepa-cyclophosphamide-fludarabine | 9 (64%) | 5 (50%) |
| Thiotepa-cyclophosphamide | 5 (36%) | 5 (50%) |
| GVHD prophylaxis | | |
| Cyclosporin A–methotrexate | 12 (85%) | 10 (100%) |
| Cyclosporin A–mycophenolate | 2 (15%) | |
| GVHD onset (day) | 48 (32–90) | |
| GVHD grade | | |
| Grade I | 3 (22%) | |
| Grade II | 8 (56%) | |
| Grade III | 3 (22%) | |
| Target organ | | |
| Skin | 8 (58%) | |
| Gut | 2 (14%) | |
| Multiple organs | 4 (28%) | |

supernatant was transferred into microcentrifuge tubes, and centrifuged a second time at 12,000*g* for 10 min at 4°C. The supernatant was removed and stored at −80°C in RNase/DNase-free tubes.

*RNA isolation*

Total RNA was extracted from 500 μL of frozen plasma samples using the miRvana isolation kit (Applied Biosystems) with minor modifications. Namely, samples were incubated 30 min at 56°C in the presence of proteinase K prior to RNA extraction to disrupt protein complexes that may sequester circulating miRNAs. Sodium dodecyl sulfate (SDS) 0.1% and 5 N acetic acid 0.06% were added to the lysate as previously described [26] to further promote the release of miRNAs from protein–nucleic acid complexes.

*MicroRNA expression profiling by qRT-PCR*

Given the lack of a suitable method for quantification of circulating microRNA, a fixed volume of total RNA was subjected to reverse transcription using a TaqMan MicroRNA Reverse Transcription Kit (No. 4366596, Applied Biosystems) and Megaplex RT primers (Human Pool A, No. 4399966, Applied Biosystems) according to the manufacturer's protocol. The cDNA libraries were pre-amplified using Megaplex PreAmp primers (Human Pool A, No. 4399233, Applied Biosystems) and PreAmp Master Mix (No. 4384266, Applied Biosystems) following the manufacturer's instructions. Subsequently, they were combined with TaqMan Universal Master Mix and loaded into TaqMan Human MicroRNA Array A (No. 4398965, Applied Biosystems), enabling accurate quantitation of 377 miRNAs selected as the miRNAs most commonly expressed by human cells. Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The threshold cycle ($C_t$) for each microfluidic card was calculated using the program SDS 2.2.2 (Applied Biosystems), setting a threshold of 0.2 and an automatic baseline from 5 to 16 cycles.

*Normalization and data analysis*

The data set was filtered by keeping only miRNAs with $C_t$ values ≤32 in all samples, reducing the number of miRNAs to be analyzed from 377 to 113. Data normalization was carried out by calculating the $\Delta C_t$ (hereafter called "ratio") between each unique pair of miRNAs in an iterative manner for each sample (e.g., $C_{tmiR1}-C_{tmiR2}$, $C_{tmiR1}-C_{tmiR3}$, $C_{tmiR1}-C_{tmiR4}$, and so on for every miRNA, thus obtaining "reciprocal ratios") [27]. Considering only those miRNAs always detected in all groups of patients (113 miRNAs), a total of 6,328 reciprocal ratios per sample were obtained (total number of ratios $= n \times (n-1)/2$, where $n$ is the number of miRNAs considered detected). Only ratios with an interquartile range (IQR) ≥0.15 were kept for further analysis. The final data set included miRNA data for 1,610 ratios from 23 samples (one sample from the aGVHD group was excluded because of poor data quality). The underlying assumption in this approach is that the ratio between two miRNA expression levels would change among groups only if one of the two is differentially expressed or if both are (e.g., one upregulated, the other downregulated) [27]. To select the most reliable differentially expressed ratios, we repeated the unpaired two-sample $t$ test with a leave-one-out cross-validation approach [28]. This consisted of repeating the statistical test $n$ times, where $n$ is the number of samples, and excluding one sample each time. Only those ratios with a nominal $p$ value $< 0.01$ in all $n$ cycles were considered statistically different. $p$ values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) method [29].

Hierarchical clustering was performed with Euclidean distance and average linkage. A supervised classification of samples was carried out using support vector machines (SVMs), as implemented in the R package "e1071" [30,31]. Linear and radial basis kernels were evaluated, with the cost and gamma (when required) parameters sampled from 0.001 to 1,000 and from $10^{-5}$ to $10^{5}$, respectively, in decimal powers. The optimal combination of parameters was found using the leave-one-out cross-validation method, with miRNAs found differentially expressed as input features. To test for overtraining and to assess the significance of the cross-validated accuracy, a nonparametric permutation test with 10,000 permutations of sample labels was performed taking into account accuracies >0.78.

To retrieve the unique deregulated miRNAs, a correlation analysis using Pearson's correlation coefficient between raw $C_t$ values from the identified ratios and the average expression level of each group (represented by the mean $C_t$ value) was performed. Accuracy, specificity, and sensitivity were computed to evaluate the performances of the classification. All statistical analyses were performed using the open-source statistical language R [32].

*Pathway analysis*

The biological role of miRNAs associated with aGVHD was investigated through the use of IPA (Ingenuity Systems, www.ingenuity.com). A $p$ value $< 0.05$ determined by Fisher's exact test was used to select statistically enriched significant results.

## Results

*Patient characteristics and aGVHD assessment*

We designed a prospective study based on the weekly collection of plasma samples from 24 lymphoma patients undergoing MUD HSCT. At the time of transplantation, all patients were in complete remission (CR).

After transplant, patients were monitored weekly for 3 months and then monthly up to 6–12 months depending on the patient's medical condition. At each visit, a complete blood cell (CBC) count; leukocyte differential count (LDC); platelet count; liver and renal function tests; drug level test; acute-phase protein monitoring; and cytomegalovirus (CMV), Epstein–Barr virus (EBV), and adenovirus monitoring were performed, but there were no significant differences between aGVHD and non-aGVHD patients (unpaired $t$ test $p > 0.05$), according to data from the literature [12].

All patients reached full donor chimerism before day 28 after transplantation, as assessed by analysis of the variable number of tandem repeats (VNTR) in peripheral blood. Platelet recovery (count >20,000/μL) was achieved at a median of day 9 post-transplantation (range 3–20); neutrophils reached values >500/μL on day 11 (range 3–17), indicating successful engraftment in all patients. Blood cell counts and immunophenotyping were

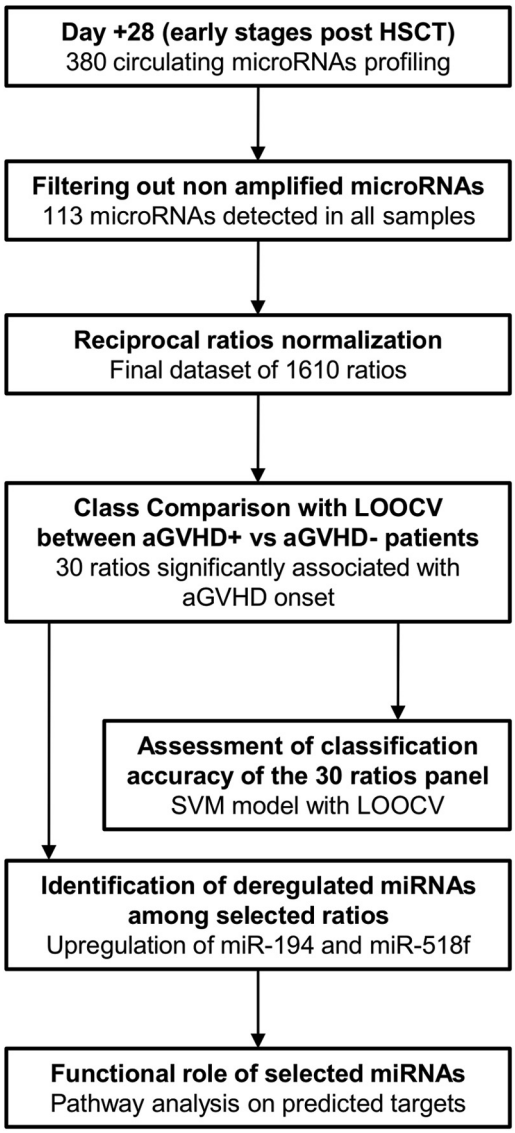


**Figure 1.** Study design.

performed on day +28 (Supplementary Table E1, online only, available at www.exphem.org) and revealed no differences between the patients developing aGVHD and those not developing it ($p > 0.05$). Defined according to the established guidelines [33], acute GVHD onset was monitored at least weekly. The overall incidence of any grade and type of aGVHD was 58% (14/24), with 8 patients developing cutaneous GVHD (grades I–III), 2 gastrointestinal GVHD (grade II), and 4 multi-organ GVHD. Patient's characteristics, treatments, and aGVHD grades and types are summarized in Table 1. As expected in a reduced intensity conditioning setting such as the one we used, all patients developed aGVHD after day 30 post-allo-HSCT (median: 48, range: 32–90). After a median follow-up of 22 months, two patients died of multiple organ failure related to aGVHD, and two died of progressive disease.

No significant correlation was found between the onset of aGVHD and donor–recipient mismatches at HLA loci, type of conditioning regimen, sex mismatch, and number of $CD34^+$ or $CD3^+$ cells in the graft (Fisher's exact test and $t$ test $p > 0.05$).

*Prospective analysis of profiling of circulating miRNAs in allografted patients*

Global miRNA analysis was performed on 240 plasma samples obtained from 24 lymphoma patients collected 1 month before allo-HSCT and from day 7 up to day 100 post-allo-HSCT. Analysis was also performed on plasma samples collected from 20 normal subjects using TaqMan Low Density Arrays (TLDA) Cards (Applied Biosystems; Fig. 1).

No significant differences were observed in the analysis of circulating miRNAs in patients before allo-HSCT and in healthy donors. In pre-HSCT samples, we were able to detect a mean of 200 miRNAs (range 152–242), and in healthy donor samples, a mean of 230 miRNAs (range 219–249). The majority of these miRNAs (68.9%) were present in both patient and donor samples at similar levels of expression (unpaired $t$ test $p$ value $> 0.05$). This is consistent with the absence of active disease at the time of transplantation for all patients.

At day +7 after allo-HSCT, a mean of 70 miRNAs of the 377 present on the card could be detected. At subsequent points, this number tended to progressively increase, probably reflecting an increase in the number of circulating cells during hematopoietic reconstitution (Table 2) [34]. The number of miRNAs detected reached values comparable to those of pre-transplantation samples between days +60 and +90 post-HSCT.

Because an ideal biomarker should be able to predict aGVHD development, we focused on miRNA data obtained at day +28, as all patients developed aGVHD after this day. At this follow-up point, the number of miRNAs detected in

**Table 2.** Prospective analysis of circulating miRNAs in allografted patients

| Time | Average number (range) of amplifiable miRNAs[a] |
|---|---|
| Healthy donors | 230 (219–249) |
| Allografted patients | |
| 1 month before HSCT | 200 (152–242) |
| +7 days post-HSCT | 70 (68–75) |
| +14 days post-HSCT | 150 (140–156) |
| +21 days post-HSCT | 181 (154–193) |
| +28 days post-HSCT | 195 (190–214) |
| +60 days post-HSCT | 213 (207–234) |
| +90 days post-HSCT | 243 (212–250) |

[a]Average number of miRNAs detected after allo-HSCT, considering miRNAs with $C_t$ values ≤32 amplifiable.

the plasma of patients was significantly lower (195) than that of healthy donors (236; mean values, $p = 4.278e\text{-}07$; Fig. 2A).

In addition, patients developing aGVHD (aGVHD group) had a significantly smaller number of circulating miRNAs compared with patients not developing aGVHD (non-aGVHD group; 182 vs. 210, respectively, mean values, $p = 0.003$; Fig. 2B). In accordance with the smaller number of miRNAs detected, these were also less abundant in the aGVHD group as indicated by higher $C_t$ values, considering all 377 miRNAs on the card (mean $C_t$: aGVHD patients, 25.8; non-aGVHD patients, 24.9; healthy donors, 23.1; Fig. 2C). Lower expression levels of circulating miRNAs were confirmed considering that only 113 miRNAs were detected ($C_t < 32$) in all samples (mean $C_t$: aGVHD, 23.9; non-aGVHD, 22.0; healthy donors, 21; $p = 0.004$; Fig. 2D).

*Profiling of circulating miRNAs before aGVHD onset identifies patients at high risk of developing the disease*

To identify miRNAs selectively expressed in one of the two conditions (aGVHD patients and non-aGVHD patients), we labeled each miRNA as "detected" ($C_t \leq 32$) or "undetected" ($C_t > 32$) in a given sample, and we determined whether detected miRNAs were statistically significantly associated with aGVHD status. Results from Fisher's exact test indicated that the presence or absence of a specific miRNA was not significantly associated with aGVHD at an FDR cutoff of 0.01. Data were normalized using a reciprocal ratio approach, as described under Methods, resulting in the $\Delta C_t$ values used as input for the subsequent analysis. Comparison of the aGVHD and non-aGVHD groups indicated that 30 ratios could clearly discriminate the two biological conditions (Figs. 1 and 3). Specifically, the 30 ratios identified with a leave-one-out cross-validation

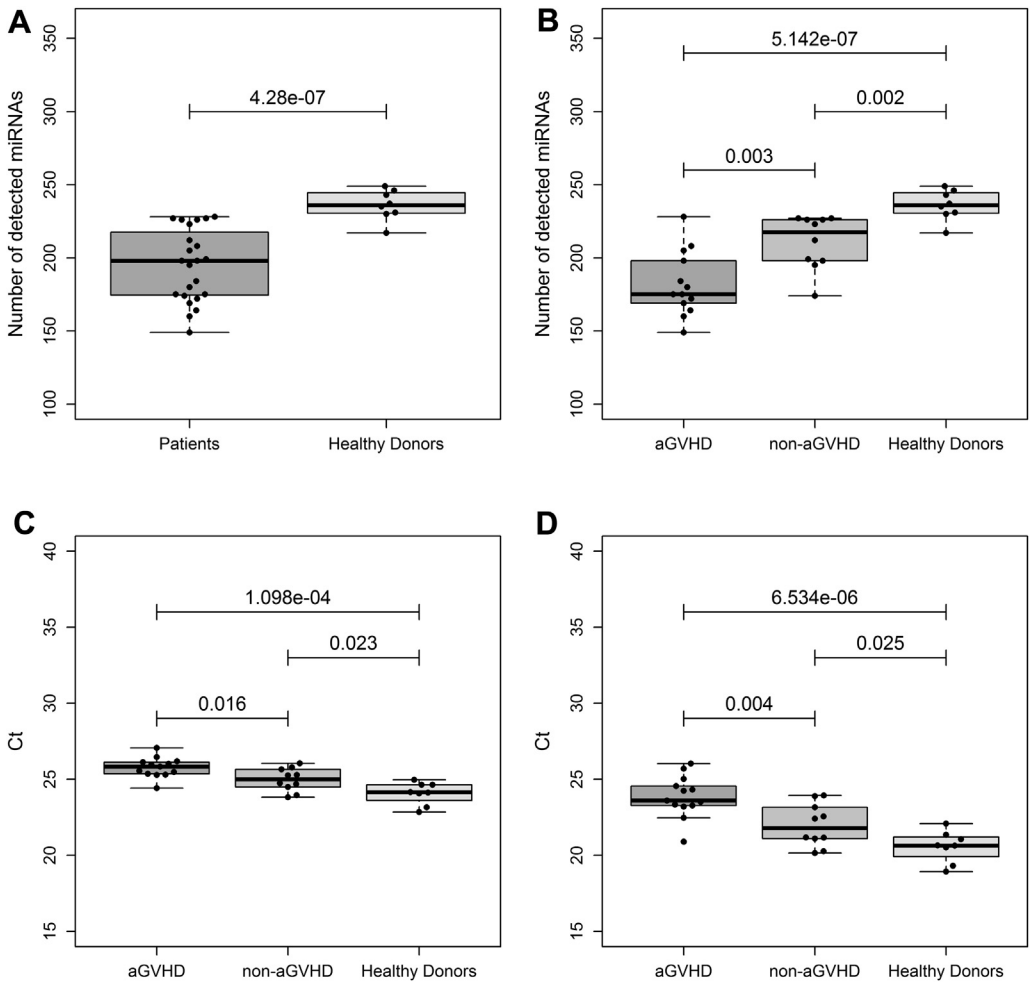


**Figure 2.** Circulating miRNAs in aGVHD patients are shifted toward lower expression levels. (**A**) The number of miRNAs expressed is significantly smaller in patients post-HSCT than in healthy donors. Moreover, the average number of miRNAs detected differs among the three biological groups. (**B**) Acute GVHD patients have a significantly smaller number of detected miRNAs compared with non-aGVHD patients and healthy donors. (**C**) Accordingly, the expression levels in the aGVHD group are lower, as indicated by higher values of cycle threshold ($C_t$), considering all 377 miRNAs on the card (mean $C_t$: aGVHD patients, 25.8; non-aGVHD patients, 24.9; healthy donors, 23.1). (**D**) These results were confirmed considering only miRNAs detected ($C_t < 32$) in all samples (mean $C_t$: aGVHD, 23.9; non-aGVHD, 22.0; healthy donors, 21). In (**A–D**), $p$ values ($t$ test) are reported.

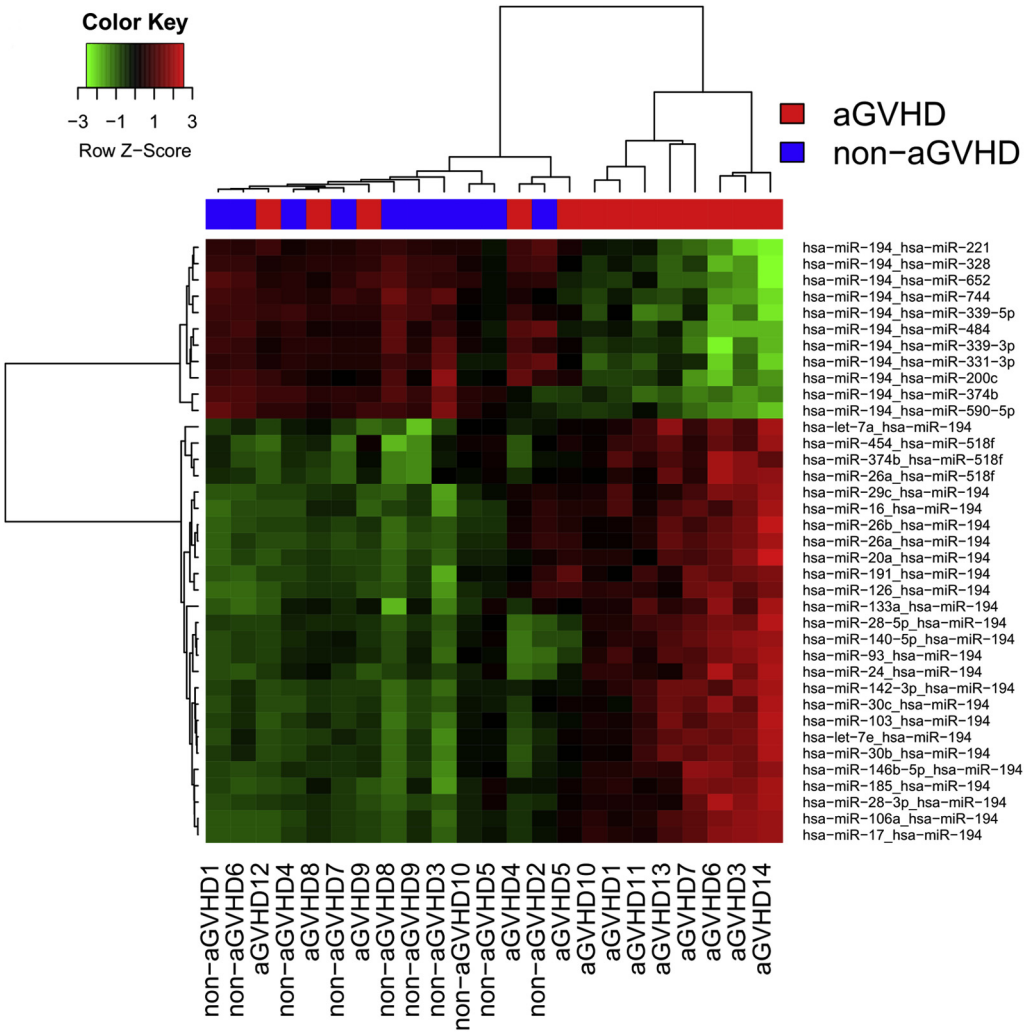


**Figure 3.** MicroRNA expression profiles can distinguish aGVHD from non-aGVHD samples. Hierarchical clustering analysis performed with the 30 ratios differentially expressed is able to discriminate aGVHD from non-aGVHD samples as expected, although some of the aGVHD patients (aGVHD12, aGVHD8, aGVHD9, aGVHD4, aGVHD5) are grouped in the non-aGVHD cluster.

class comparison approach [28] (Table 3) were used to perform a hierarchical clustering analysis that was able to clearly separate aGVHD and non-aGVHD patients, with the exception of 5 aGVHD cases that presented an expression profile closer to that of the non-aGVHD group (Fig. 3).

*Identified miRNA panel discriminates aGVHD from non-aGVHD patients with high accuracy*

As the final goal of circulating miRNA studies is to develop a diagnostic or prognostic predictor, we used SVMs to evaluate the ability of the panel of 30 ratios previously identified to discriminate between aGVHD and non-aGVHD samples in a supervised manner. The list of 30 ratios was used directly as input to build the SVM model. The best trained model indicates that the 30 ratios obtained by analyzing the plasma samples before aGVHD onset can predict the development of aGVHD with an accuracy of 0.78, a specificity of 1, and a sensitivity of 0.62 (cross-validated accuracy; $p$ value $= 9 \times 10^{-4}$). Four aGVHD patients were misclassified and erroneously assigned to the non-aGVHD class (Fig. 4). Of note, three of them had grade I aGVHD.

*miR-194 and miR-518f are overexpressed in aGVHD samples*

After the normalization step, we considered the 27 unique miRNAs involved in each of the 30 significant ratios to determine which of them was actually deregulated in the aGVHD class.

We performed a correlation analysis using Pearson's correlation coefficient between the raw $C_t$ values of the miRNAs present in the 30 differentially expressed ratios and the average expression levels of aGVHD and non-aGVHD samples, and only miR-194 and miR-518f were not significantly correlated with the average expression value (miR-194: $r = 0.27$, $p = 0.21$; miR-518f: $r = 0.09$,

**Table 3.** Leave-one-out cross-validated class comparison: miRNA ratios differentially expressed among aGVHD and non-aGVHD patients[a]

| Ratio | Fold change | *p* value | FDR |
|---|---|---|---|
| hsa-miR-194_hsa-miR-374b | −1.13 | 0.0002 | 0.0597 |
| hsa-miR-194_hsa-miR-590-5p | −1.12 | 0.0004 | 0.0597 |
| hsa-miR-194_hsa-miR-652 | −1.13 | 0.0006 | 0.0597 |
| hsa-miR-26b_hsa-miR-194 | 1.15 | 0.0007 | 0.0597 |
| hsa-miR-194_hsa-miR-744 | −1.11 | 0.0008 | 0.0597 |
| hsa-miR-26a_hsa-miR-194 | 1.16 | 0.0009 | 0.0597 |
| hsa-miR-106a_hsa-miR-194 | 1.13 | 0.0010 | 0.0597 |
| hsa-miR-16_hsa-miR-194 | 1.15 | 0.0011 | 0.0597 |
| hsa-miR-17_hsa-miR-194 | 1.14 | 0.0011 | 0.0597 |
| hsa-miR-29c_hsa-miR-194 | 1.11 | 0.0013 | 0.0597 |
| hsa-miR-20a_hsa-miR-194 | 1.13 | 0.0013 | 0.0597 |
| hsa-miR-194_hsa-miR-484 | −1.12 | 0.0014 | 0.0597 |
| hsa-miR-142-3p_hsa-miR-194 | 1.13 | 0.0014 | 0.0597 |
| hsa-miR-126_hsa-miR-194 | 1.11 | 0.0016 | 0.0597 |
| hsa-miR-185_hsa-miR-194 | 1.12 | 0.0017 | 0.0597 |
| hsa-miR-194_hsa-miR-339-3p | −1.12 | 0.0018 | 0.0597 |
| hsa-miR-103_hsa-miR-194 | 1.14 | 0.0019 | 0.0597 |
| hsa-miR-194_hsa-miR-328 | −1.12 | 0.0021 | 0.0647 |
| hsa-miR-146b-5p_hsa-miR-194 | 1.12 | 0.0022 | 0.0647 |
| hsa-miR-28-3p_hsa-miR-194 | 1.11 | 0.0023 | 0.0647 |
| hsa-miR-191_hsa-miR-194 | 1.12 | 0.0024 | 0.0647 |
| hsa-miR-194_hsa-miR-339-5p | −1.14 | 0.0026 | 0.0651 |
| hsa-miR-30c_hsa-miR-194 | 1.13 | 0.0028 | 0.0668 |
| hsa-miR-133a_hsa-miR-194 | 1.14 | 0.0029 | 0.0668 |
| hsa-let-7e_hsa-miR-194 | 1.13 | 0.0029 | 0.0668 |
| hsa-miR-30b_hsa-miR-194 | 1.13 | 0.0030 | 0.0674 |
| hsa-miR-194_hsa-miR-331-3p | −1.10 | 0.0035 | 0.0691 |
| hsa-let-7a_hsa-miR-194 | 1.16 | 0.0037 | 0.0719 |
| hsa-miR-374b_hsa-miR-518f | 1.13 | 0.0041 | 0.0730 |
| hsa-miR-454_hsa-miR-518f | 1.11 | 0.0042 | 0.0730 |

[a]Thirty ratios selected with the leave-one-out cross-validation class comparison ($p < 0.01$). Fold change, *p* value, and false discovery rate (FDR) were calculated using all samples.

$p = 0.68$). Of note, both were upregulated in subjects experiencing aGVHD, suggesting a possible role for the two miRNAs in aGVHD development, whereas the other miRNAs present in the 30 ratios were stable and can be considered housekeeping molecules. TaqMan array data were also confirmed by single-assay RT-PCR.

### *Pathway analysis reveals regulatory role of miR194 and miR518f in biological processes associated with aGVHD pathogenesis*

Investigation of the biological role of miRNAs is a challenging task that requires the identification of target genes and the classification of these genes into functional categories or known pathways. Ingenuity Pathway Analysis (IPA) was used to identify both miRNA target mRNAs and the biological processes in which miR-194 and miR-518f are involved. IPA revealed several possible pathways altered at the transcriptional level or potentially modified by miRNAs at the post-transcriptional level. Among the targeted mRNAs, we could identify key molecules of the JAK-STAT pathway, MAPK signaling, and NFKB pathway that

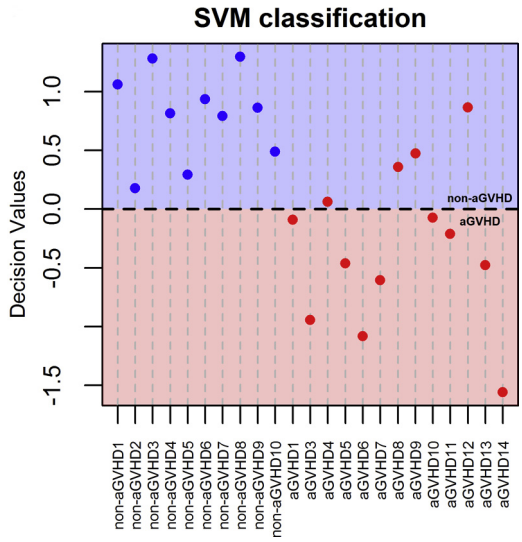


**Figure 4.** MicroRNA ratios and aGVHD prediction. SVM classification of samples based on the 30 differentially expressed ratios. *Dots* represent the decision values assigned by the SVM algorithm to each patient's miRNA profile at day +28 post-HSCT. The decision value represents the confidence that an SVM classifier has in classifying a given sample. A positive value assigns the sample to the non-aGVHD class, a negative value to the aGVHD class.

are well known for their role in aGVHD pathogenesis [35,36] (Fig. 5B, C). Moreover, IPA analysis revealed "Wnt/β-catenin signaling" as the top canonical pathway (Fig. 5A), suggesting that deregulation of this signaling might play a key role in aGVHD pathogenesis [37].

## Discussion

We investigated changes in the levels of circulating miRNAs after allo-HSCT in a selected cohort of lymphoma patients receiving grafts from MUDs and correlated them with the occurrence of aGVHD. Our study indicates that the analysis of circulating miRNAs at early points after HSCT is feasible and can be performed using a simple and broadly applicable technique such as qRT-PCR amplification. Comparison of pre-HSCT miRNA profiles with those of healthy subjects did not reveal significant differences either in the number of miRNAs detected or in the levels of expression, in accordance with the absence of active disease in our patients at the time of transplantation. After HSCT, the number of detectable circulating miRNAs is lower than that of healthy donors, but increases progressively with time. This may reflect the immune reconstitution process that physiologically occurs after transplantation in which the number of circulating cells gradually increases to reach the levels of healthy subjects [34].

When attention is focused on day +28 post-allo-HSCT, global miRNA expression profiling was able to discriminate subjects who later developed aGVHD in our patient cohort. However, when considering the use of circulating miRNAs

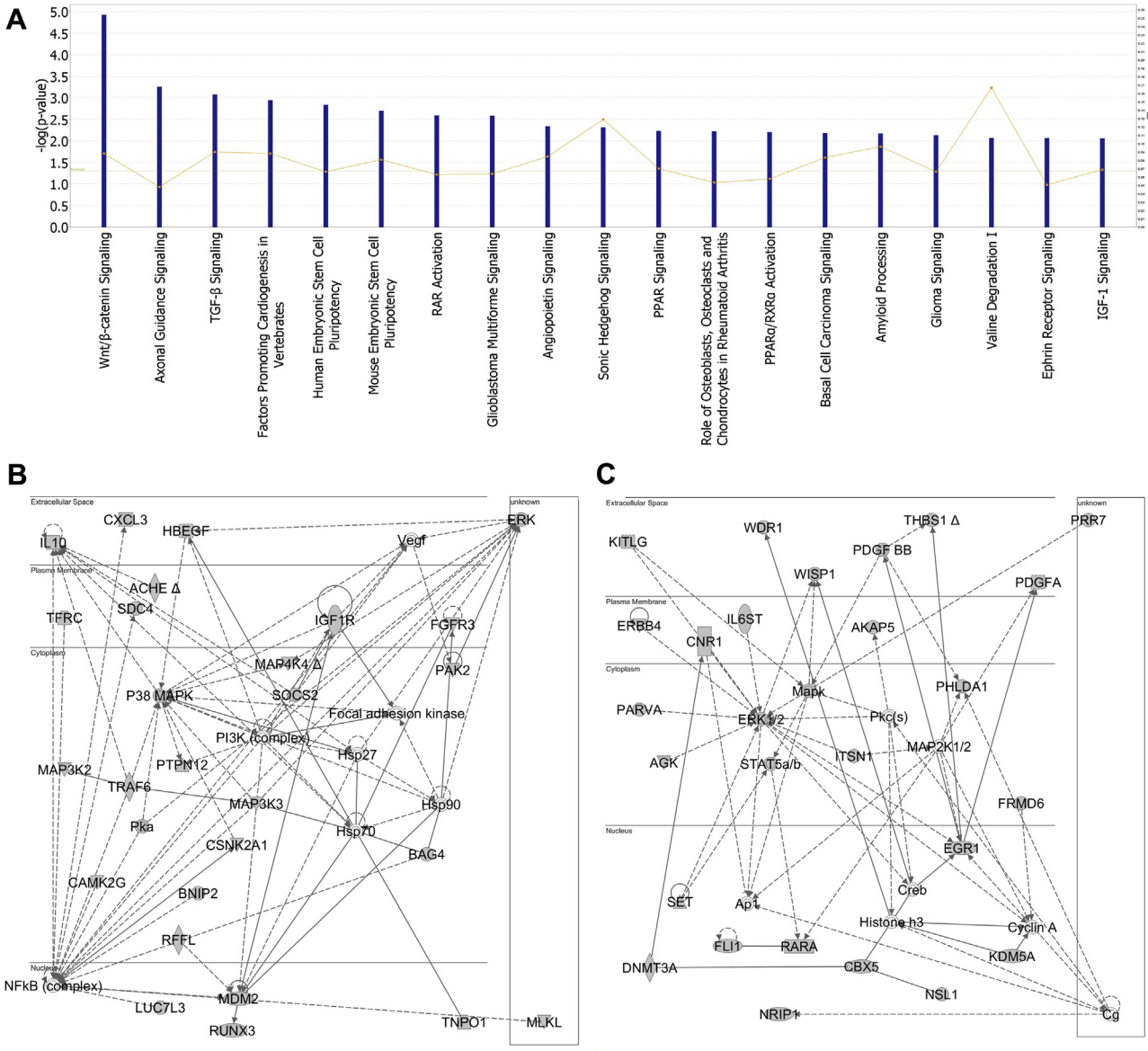


**Figure 5.** Pathway and network analysis of miR-194 and miR-518f target genes performed with the IPA software. Global canonical pathway analysis revealed that target genes of miR-194 and miR-518f are involved in pathways that could be related to aGVHD. (**A**) Data for canonical pathways with a $p$ value $< 0.01$, calculated using Fisher's exact test, are shown. (**B, C**) IPA analysis of miRNA target mRNAs: (**A**) graphical presentation of "Infectious Disease, Cell Death and Survival, Cellular Growth and Proliferation" network (score 32, focus molecules 25); (**C**) Graphical presentation of "Cellular Development, Embryonic Development, Hematological System Development and Function" network (score 30, focus molecule 24).

as biomarkers, some aspects must be taken into account: (i) the accuracy of circulating miRNA profiling is wholly reliant on sample type, processing, and profiling method; (ii) analytical variables, such as data normalization, have the potential to bias results [38]; (iii) there is no validated technique to quantify plasma miRNAs; and (iv) little concordance is observed between similar and independent studies [24,38].

Data normalization is a major challenge when analyzing qRT-PCR results. Different strategies have been proposed [39] such as the use of synthetic miRNA molecules as spike-in controls to normalize expression levels of target miRNAs. These molecules, although useful in assay validation and quality control [40], do not take into account differences in template quality or efficiency of reverse transcription reactions. In previous studies, miRNA expression was estimated by relative quantification using RNU6, RNU6B, 18S, and miR-16 as internal controls [20]. However, the stability of these molecules in plasma is controversial, and in our study, many of the so-called invariant miRNAs were almost undetectable. Selection of stable miRNAs by algorithms designed to evaluate the variability of candidate housekeeping miRNAs across samples is an alternative approach to identify potential normalizers [41,42], together

with the global mean normalization method [43]. These global normalization approaches are based on the assumption that the same total amount of miRNAs is expected in all samples and that only a small fraction of miRNAs is deregulated. When profiling circulating miRNAs, these assumptions should be viewed cautiously as miRNAs exhibit great variability in expression levels [44].

Consequently, although widely used in the literature, these normalization methods are not ideal when analyzing circulating miRNA profiling data. This implies that further research is needed to identify suitable methods, and in this context, a ratio-based approach has been proposed [27] in which all miRNAs are used, in turn, as housekeeping. This method has the advantage of limiting a priori assumptions, although high redundancy is generated, and the results from raw and normalized data should be interpreted together to determine which miRNA has a relevant role and which functions as a calibrator.

In the absence of a consensus and taking into account all the pros and cons of the aforementioned normalization methods, we tested all of them. Regardless of the normalization approach used, the circulating miRNA profiles were able to discriminate patients that will later develop aGVHD. Bearing in mind the limitations of each method, the reciprocal ratio approach was used to generate the data in this study, as this approach overcomes issues regarding quantity and quality of the starting material.

Comparison of the aGVHD and non-aGVHD classes revealed that 30 ratios could clearly discriminate the two biological conditions. Moreover, two unique miRNAs (miR-194 and miR-518f) were found to be upregulated in samples of patients who would later develop aGVHD with respect to those not experiencing the complication. Of interest, pathway analysis allowed for the identification of 389 target genes for miR-194, and 34 target genes for miR-518f. Among miR-518f target genes, we found EGR-1, a transcription factor that modulates the expression of several genes involved in inflammation, neutrophil and monocyte adhesion and chemotaxis, tissue repair, regeneration, and development [45,46]. Moreover, miR-194 targets include SOCS2, a key molecule of the JAK-STAT pathway; MAPK family genes that are involved in MAPK signaling, TCR signaling, and the NFKB pathway; and CXCL3, chemokine ligand 3, a molecule involved in cytokine–cytokine receptor interaction and chemokine signaling, a crucial step in aGVHD pathogenesis [47]. In addition, among the top significantly represented canonical pathways were Wnt/β-catenin signaling, TGFβ signaling, and Toll-like receptor signaling. Wnt/β-catenin signaling has been reported to play an important role in gastrointestinal epithelial regeneration after chemotherapy-induced damage [37]. The TGFβ pathway is an important modulator of immune responses, with direct consequences for GVHD pathophysiology [48]. Toll-like receptors (TLRs) are important components of innate immunity and are known for sensing various microbial ligands or danger signals (such as those subsequent to a conditioning regimen), especially in the gut. Taken together, these results suggest that targeting and subsequent downregulation of genes involved in the aforementioned pathways might be of interest in a GVHD context.

To date, only one study by Xiao et al. has analyzed plasma miRNAs as potential biomarkers for aGVHD using a similar qPCR platform [24]. Of note, elevated levels of four miRNAs were detected before aGVHD diagnosis. However, when we compared our results with those obtained by Xiao et al., there was no consistency. There are several possible reasons for the lack of reproducibility between the two studies:

1. Patient characteristics: Xiao et al. analyzed plasma samples from a heterogeneous group of patients, including patients with diverse disease types at the time of HSCT (both malignant and not) who underwent related or unrelated HSCT after a reduced-intensity or myeloablative conditioning regimen. In contrast, our study focused on the analysis of a homogeneous sample group of 24 lymphoma patients undergoing reduced-intensity, conditioning-unrelated HSCT. All patients enrolled in our study were in complete remission at the time of transplantation; therefore, we can exclude differences caused by a primary malignancy. Moreover, we could not find any differences among the subgroups of lymphoma patients (Hodgkin's and non-Hodgkin's), suggesting that the differences found at day +28 post-HSCT can be truly associated with aGVHD onset.
2. Sample preparation and miRNA profiling technique: As outlined in a very recent review [49], a great source of variability in miRNA assessment of body fluids is represented by both the extraction methodology and the analysis platform employed. Even though the sample collection and extraction methodology used by Xiao et al. are comparable to ours, this is not true of the profiling method. Xiao et al. used a SYBR green-based qPCR platform, whereas we took advantage of a TaqMan-based qPCR platform. The SYBR green-based qPCR platform lacks specificity, while probe-based chemistry qPCR provides several benefits over dye-based technologies. The TaqMan low-density array used in our study combines high levels of specificity and reproducibility, making it one of the best choices for quantification of circulating miRNAs.
3. Normalization strategy: Xiao's group employed a traditional housekeeping gene normalization, using Cel-miR-39 as endogenous control. On the other hand, we decided to normalize our data by calculating reciprocal ratios of the miRNA expression levels, thus overcoming differences in template quantity and quality.

This preliminary study confirms that profiling of circulating miRNAs early after allo-HSCT is feasible and could be used to monitor aGVHD onset. Although our results appear promising, we realize several concerns over the use of circulating miRNAs as biomarkers: (i) the accuracy of circulating miRNA profiling is wholly reliant on sample type, processing, and profiling method; (ii) analytical variables, such as data normalization, have the potential to bias results [38]; (iii) there is no validated technique to quantify plasma miRNAs; (iv) frequently, little concordance is observed between similar and independent studies [38].

One could obviously argue that the limited number of patients included in our study as well as the diverse conditioning regimens and patient characteristics might account for the different results. The lack of concordance with previous works [24,38,50] also points to the need for standardization of preanalytical and analytical variables in circulating miRNA studies.

In conclusion, we believe that the utility of circulating miRNAs in aGVHD prediction, diagnosis, and prognosis is still questionable. Standardization and reproducibility remain the first goals to be accomplished before miRNAs are introduced as biomarkers in clinical practice.

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## Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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**Supplementary Table E1.** Blood cell counts at day +28 post-HSCT[a]

| Cell population | Mean count, No./μL (range) | | *p* value |
|---|---|---|---|
| | aGVHD group | Non-aGVHD group | |
| White blood cells | 4,550 (1,450–8,350) | 3,150 (2,730–3,570) | 0.55 |
| Lymphocytes | 425 (200–800) | 550 (300–700) | 0.59 |
| Erythrocytes | $3.3 \times 10^6$ (2.7–3.8) | $3.1 \times 10^6$ (2.7–3.4) | 0.71 |
| Neutrophils | 2,600 (1,600–4,000) | 1,700 (1,500–3,000) | 0.41 |
| Platelets | $211.7 \times 10^3$ (44–417) | $235.5 \times 10^3$ (130–336) | 0.86 |
| $CD3^+$ | 59.72 (4.7–117.6) | 107.5 (88.3–128.5) | 0.3 |
| $CD3^+/CD4^+$ | 21.19 (3.2–55.5) | 36.86 (28.7–45.1) | 0.45 |
| $CD3^+/CD8^+$ | 30.8 (1.38–72.1) | 51.92 (37.1–66.7) | 0.42 |
| $CD56^+/CD16^+$ | 114.2 (14.54–223.68) | 257.2 (156.4–359) | 0.23 |
| $CD3^+/CD56^+$ | 8.57 (0.1–31.6) | 13.4 (2.6–55.8) | 0.74 |
| $CD14^+$ | 2,112 (2.6–4,675) | 1,362 (110.4–3,263) | 0.7 |

[a]Routine blood tests are reported along with immunophenotyping results. Mann-Whitney test was performed to assess differences between the aGVHD and non-aGVHD groups.