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# Multivariate Analysis of Immune Reconstitution and Relapse Risk Scoring in Children Receiving Allogeneic Stem Cell Transplantation for Acute Leukemias

Manuela Spadea, MD,<sup>1</sup> Francesco Saglio, MD,<sup>1</sup> Serena I. Tripodi, MD,<sup>2</sup> Mariacristina Menconi, MD,<sup>3</sup> Marco Zecca, MD,<sup>2</sup> and Franca Fagioli, MD<sup>1</sup>

**Background.** A timely and effective immune reconstitution after hematopoietic stem cell transplantation (HSCT) is of crucial importance to enhance graft-versus-leukemia reaction in hematological malignancies. Several factors can influence the yield of this process, and new mathematical models are needed to describe this complex phenomenon. **Methods.** We retrospectively analyzed immune reconstitution in the early post-HSCT period in a multicenter cohort of 206 pediatric patients affected by acute lymphoblastic leukemia, acute myeloblastic leukemia, and myelodysplastic syndrome who received their first allo-HSCT. All patients were in complete morphological remission at transplantation and were followed-up at least 26 mo post-HSCT. Blood samples for analysis of lymphocyte subset numbers were collected at day 100 ( $\pm 20$  d). **Results.** The 2-y cumulative incidence of relapse was 22.2% (95% confidence interval [CI], 17.3-27). Using principal component analysis, we identified based on 16 input variables a new multivariate model that enables patients' description in a low-dimensional model, consisting of the first 2 principal components. We found that the numbers of CD3<sup>+</sup>/CD4<sup>+</sup>/CD8<sup>+</sup> lymphocyte subsets at day 100 post-HSCT and acute graft-versus-host disease had the greatest impact in preventing relapse. We ultimately derived a risk score defining high- or medium-low-risk groups with 2-y cumulative incidence of relapse: 35.3% (95% CI, 25.6-45) and 15.6% (95% CI, 10.1-20.7), respectively ( $P=0.001^*$ ). **Conclusions.** Our model describes immune reconstitution and its main influencing factors in the early posttransplantation period, presenting as a reliable model for relapse risk prediction. If validated, this model could definitely serve as a predictive tool and could be used for clinical trials or for individualized patient counseling.

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) plays a major role in the treatment of patients at a high risk for both pediatric acute lymphoblastic leukemia (ALL)<sup>1</sup> and acute myeloblastic leukemia (AML).<sup>2</sup> Although significant improvement in nonrelapse mortality has been registered over time, relapse risk did not vary, making relapse the major cause of treatment failure.<sup>3</sup> From

this standpoint, HSCT offers the important advantage of the immune-mediated clearance of recipient leukemic cells by donor lymphocytes (graft-versus-leukemia reaction).<sup>4</sup> Indeed, the early posttransplant period is characterized by multiple immune defects, and the restoration of the immune system post-HSCT is, therefore, one of the main factors influencing outcomes post-HSCT.<sup>5-8</sup> Thus, enhancing this process is an area of intensive research.<sup>9,10</sup> Multiple factors influence immune reconstitution post-HSCT:

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<sup>1</sup> Pediatric Oncohematology, Stem Cell Transplantation and Cell Therapy Division, A.O.U. Città della Salute e della Scienza-Regina Margherita Children's Hospital, Turin, Italy.

<sup>2</sup> Pediatric Hematology-Oncology, Fondazione Istituti di ricovero e cura a carattere scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy.

<sup>3</sup> Haematopoietic Stem Cell Transplantation Unit, Paediatric Clinic, University Hospital of Pisa, Pisa, Italy.

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article. All the authors participated in writing the final version of the article. F.F. and M.Z. critically revised the article.

Correspondence: Manuela Spadea, MD, Pediatric Oncohematology, Stem Cell Transplantation and Cell Therapy Division, A.O.U. Città della Salute e della Scienza, Regina Margherita Children's Hospital, University of Turin Piazza Polonia 94, Torino 10126, Italy. (manuela.spadea@gmail.com).

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recipient age, donor characteristics (eg, -matched/mismatched), graft composition (eg, in vivo or ex vivo T-cell depletion), conditioning regimen, the occurrence of acute graft-versus-host disease (aGvHD) and its treatment (eg, steroid administration) and prophylaxis, or the occurrence of infections (bacterial, viral, or fungal).<sup>11</sup> These factors are strictly interconnected and valuable analysis of their interactions in the immune reconstitution process requires new multivariate statistical models,<sup>12</sup> which could represent this complex phenomenon as a whole. Indeed, an effective description of this process ultimately allows patients' classification in lower or higher risk groups, with regard to immune reconstitution,<sup>12</sup> potentially opening new perspectives for tailored therapeutic interventions, with the aim of enhancing the immune reconstitution itself and preventing relapse occurrence.

In this multicentric study, we generated a new multivariate model describing immune reconstitution and its main influencing factors by day 100 post-HSCT in a pediatric cohort with hematologic malignancies. We ultimately identified 2 groups of patients with regard to relapse risk (high or medium-low) and proposed a new easy-to-use tool that could eventually be used as a predictive model for clinical and research purposes.

## MATERIALS AND METHODS

### Patients

This multicentric, retrospective, cohort study included 206 consecutive patients (122 males, 84 females; median age 8.57 y, interquartile range 9.15) who received their first allogeneic HSCT at the Regina Margherita Children's Hospital, Turin, Italy; San Matteo Children's Hospital, Pavia, Italy; and Pisa University Hospital, Pisa, Italy, for acute leukemias between January 1, 2012, and December 31, 2018. Patients who experienced primary graft failure, death, or relapse within 100 d post-HSCT were excluded. All data were retrieved retrospectively from clinical records according to the policy approved by our Institutional Committee on Medical Ethics and after obtaining informed consent from parents or legal guardians.

The clinical characteristics of the 206 patients are summarized in Table 1. The conditioning regimens varied according to the underlying disease and center-specific protocols. More specifically, total body irradiation was used in 117 cases, whereas chemotherapy was used in the remaining ones (Table 1).

We included patients who received bone marrow (n=136) or peripheral blood stem cells (n=60) or cord blood (n=10) as stem cell sources. HLA typing was performed using high-resolution allelic typing at HLA-A, -B, -C, -DRB1, and -DQB1 loci. Matched sibling donors were defined as genotypically or phenotypically identical siblings (n=28). In the absence of matched sibling donors, 9 of 10 or 10 of 10 mismatched unrelated donors and matched unrelated donors, respectively, were recruited from national and international donor registries (n=115). In the absence of matched donors, haploidentical family donors were chosen (n=63). Graft-versus-host disease (GvHD) prophylaxis was administered according to the donor type and stem cell source and consisted either of cyclosporine A or tacrolimus (target trough levels 100–220 ng/mL and 5–10 ng/mL, respectively). It was maintained at least for a period of 3 mo before tapering. Rabbit antihuman thymocyte globulins (ATG; Grafalon Neovii 5 mg/kg/d or thymoglobuline, Sanofi 3.5 mg/kg/d, from day -4 to -2)

**TABLE 1.**  
Patients' characteristics

Clinical characteristic		(N = 206)
Sex	Male	122 (60%)
	Female	84 (40%)
Age at transplantation	Median	8.57 (interquartile range, 9.15)
Disease	ALL	121 (59%)
	AML	64 (31%)
	MDS	21 (10%)
Disease status at transplantation	CR 1	90 (44%)
	CR 2	80 (39%)
	≥CR 3	15 (7%)
	Persistent disease	21 (10%)
Donor	MSD	28 (14%)
	MUD	79 (38%)
	MMUD	36 (17%)
	Haploidentical	63 (31%)
Source	PBSC	60 (29%)
	BM	136 (66%)
	UCB	10 (5%)
Conditioning regimen	TBI-based	117 (57%)
	Chemotherapy alone	89 (43%)
GvHD prophylaxis	ATG-MTX-CSA	107 (52%)
	CSA±MTX	28 (14%)
	ATG-PDN-CSA	8 (4%)
	PT-Cy-FK506-MMF	19 (9%)
	αβ <sup>+</sup> /CD19 <sup>+</sup> negative selection-ATG-rituximab	44 (21%)
Acute GvHD	None	106 (52%)
	I–II	87 (42%)
	III–IV	13 (6%)
Bacterial infections		67 (32%)
Viral infections		127 (62%)
Fungal infections		17 (8%)
Steroid therapy		89 (43%)

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATG, antihuman thymocyte globulin; BM, bone marrow; CR, complete remission; CSA, cyclosporine A; GvHD, graft-versus-host disease; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MMUD, mismatched unrelated donor; MSD, matched sibling donor; MTX, methotrexate; MUD, matched unrelated donor; PBSC, peripheral blood stem cells; PDN, prednisolone; PT-Cy, posttransplantation cyclophosphamide; TBI, total body irradiation; UCB, unrelated cord blood.

were administered in 159 patients. In all cases in which an unrelated donor was chosen, a short course of methotrexate was added. Prednisolone was used as GvHD prophylaxis in patients receiving an unrelated cord blood transplant. Among patients receiving haploidentical transplantation, 19 were treated with a high dosage of cyclophosphamide (total dose 100 mg/kg) on days +3 and +4 post-HSCT, whereas patients receiving an alpha/beta depleted haploidentical transplantation did not receive any post-HSCT immunosuppression.

All patients affected by AML and ALL were in complete morphological remission at the time of HSCT. All patients undergoing clinical and hematological assessments both before and after transplantation were followed-up for at least 26 mo post-HSCT, according to our Centers Standard Operating Policies. Regular blood samples for analysis of lymphocyte subset numbers were collected at day 100 (±20 d).

In our cohort, during the first 100 d posttransplantation, 67 (32%) patients presented with bacterial infection, 127 (62%) with viral infection, and 17 (8%) had a fungal infection. aGVHD was diagnosed in 100 patients (48%). Among them,

13 patients presented grade III–IV aGVHD (13%). Systemic corticosteroids were administered in 89 (43%) patients.

### Definitions and Endpoints

The primary endpoint of this study was to define a risk score of overt relapse in children affected by acute leukemias, through the use of a principal component analysis (PCA)-based low-dimensional model describing the complex relationship between all the major common variables related to immune reconstitution post-HSCT. The ultimate goal was to provide an objective and easy-to-use tool that can be used to associate a novel patient at day +100 post-HSCT to a class at risk of relapse, using clinical and easily available variables.

Bacterial infections were considered when microorganisms were isolated from  $\geq 1$  blood culture during the first 100 d posttransplantation.

Viral infections were considered when positive DNAemia was detected in blood samples 1 or more times by quantitative polymerase chain reaction for routine analysis or in other biological samples if patients had concurrent symptoms of infection during the first 100 d posttransplantation. All patients underwent viral reactivation monitoring at least 2 times a week (Epstein-Barr virus, cytomegalovirus and adenovirus) as clinical routine until cessation of immunosuppression, but at least until day 100.

Fungal infections were considered when proven or probable cases were documented during the first 100 d posttransplantation, according to the consensus definitions developed by the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group.<sup>13</sup>

All patients received antiviral and antifungal prophylaxis post-HSCT at least until 4 wk after cessation of immunosuppression, according to our Centers Standard Operating Procedures.

Steroid therapy was considered when a dosage of at least 1 mg/kg/d of systemic corticosteroids was administered.

aGVHD was diagnosed and graded according to standard criteria.<sup>14</sup>

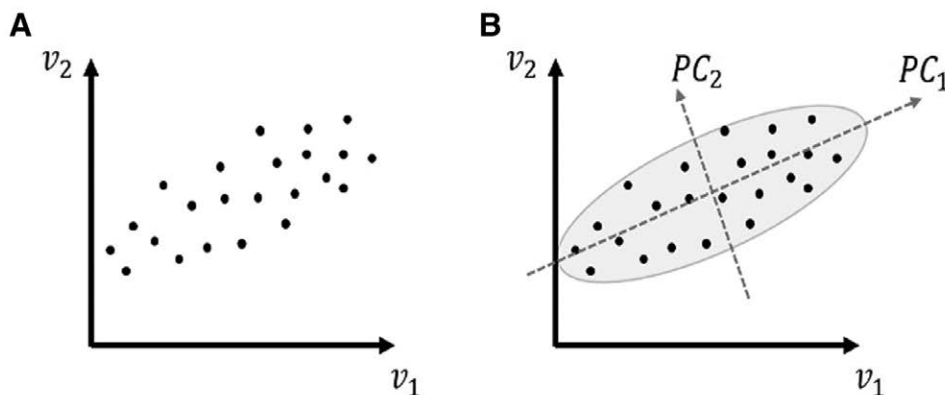
Relapse incidence (RI) is defined as the probability of having had a relapse. If the patient died without experiencing relapse or is still alive by the end of the study, data are censored on the date of death or on the date of the last follow-up, respectively.

Immune recovery was investigated by multicolor flow cytometry on peripheral blood at day 100 post-HSCT and included the absolute enumeration of total T cells ( $CD3^+$ ), helper T cells ( $CD3^+CD4^+$ ), cytotoxic T cells ( $CD3^+CD8^+$ ), natural killer cells ( $CD16^+CD56^+$ ), and B cells ( $CD19^+CD20^+$ ).

### Multivariate Model

With the ultimate goal of identifying a low-dimensional model of HSCT immune reconstitution, we used a statistical method named PCA.<sup>15</sup> PCA is a statistical tool commonly used to infer from data a mathematical model that, given a set of  $N$  subjects characterized by  $k$  variables, provides a measure of the impact of each single variable with respect to the others, in an exhaustive manner on the investigated outcome. In other words, the algorithm takes as inputs the measures of a selected number of variables (listed below) and provides as output the combination of them that explains the larger variability in the data set. A simple example with 2 variables only is given in Figure 1. The most “important” in terms of variance that it can explain will be named first principal component (PC1), and represents the combination of variables characterized by the maximum variability in the data set (ie, it explains the maximum percentage of variance in the data set). Subsequently, we identified other principal components (PCs) (total number of PCs equal to the original numbers of  $k$  variables), which, in turn, explain a reduced amount of variability. The combination of all the  $k$  PCs provided by PCA describes the 100% of the total data set variability, and (PCs) are defined hierarchically following the relevance of each of them for the explanation of the phenomenon. For this reason, the first 2 PCs are the backbones for a low-dimensional representation. Each element of the data set, described by the  $k$  variables, is characterized by a set of coefficients, 1 for each PC considered, which identifies the positioning of the subject in the PCs plane (see Figure 1).

We considered in this study a grand total of 16 variables, namely: (1) the type of leukemia (ALL, AML, myelodysplastic syndrome), (2) the disease status, (3) donor–recipient HLA-matching, (4) total body irradiation (TBI), (5) ATG, (6) posttransplantation cyclophosphamide, (7) aGVHD, (8) bacterial infections, (9) viral infections, (10) fungal infections, (11) steroids, (12)  $CD3^+$  absolute values, (13)  $CD3^+CD4^+$  absolute values, (14)  $CD3^+CD8^+$  absolute values, (15)  $CD16^+CD56^+$  absolute values, and (16)  $CD19^+CD20^+$



**FIGURE 1.** A, an example with 2 variables,  $v_1$  and  $v_2$ , and a number of subjects reported as black dots. B, the output of the PCA. The method identifies PC1 and PC2, which represent the optimal combination of  $v_1$  and  $v_2$  so that PC1 is orientated along the maximum data distribution; PC2 is orthogonal to PC1 and orientated along the maximum data distribution. If we rotate the data in the new coordinates defined by PC1 and PC2, we have that most of the differences between the subjects of this example are indeed described by PC1 only, and, therefore, this represents a low-dimensional model because 1 single variable (PC1) represents a good approximation of the original model composed by 2 variables ( $v_1$  and  $v_2$ ). PC, principal component; PCA, principal component analysis.

absolute values. The choice of these variables was based on literature evidence regarding immune reconstitution and its main influencing factors.<sup>11</sup> Lymphocyte subset counts were normalized with regard to the age range of each patient, as reported by Comans-Bitter et al<sup>16</sup> to compensate for age dependencies. We found 3 patients whose lymphocyte subset numbers, after normalization, were higher than the median plus 5 times the interquartile range. These patients were considered as outliers and were removed from the data set. Then, data were normalized to have unit variance, such that variables with a higher range of variations do not dominate over the others.

The 203 patients were then fed into the PCA algorithm<sup>15</sup> to identify the PCs and the relative scores and to obtain the representative eigenvectors. Each one of the  $k$  original variables contributes to the definition of the PCs (see Figure 2): the longer is the length of the segment, the larger is the contribution of the single variable. Each subject is represented in the PCs plane by a single dot, which is marked red if the patient presented relapse and green otherwise (see Figure 3). It is possible to observe a higher concentration of red dots in the low-left quadrant of Figure 3. For this reason, we targeted the identification of 3 zones associated with 3 different potential risk scores (see Figure 4). To do this, we used the following procedure: as the first step, we calculated the arithmetical mean of the relapsed and nonrelapsed patients (red and green dots, respectively). As the second step, we calculated the line equidistant from the 2 mean values (ie, following the generation of Voronoi plot rules<sup>17</sup>). This line, depicted in Figure 4 as a

dashed red line, represents the threshold in the PCs' plane that maximizes the separation of relapsed and nonrelapsed patients in the plot. Finally, we identified the second boundary (green dashed line in Figure 3) by mirroring the first boundary with respect to the origin of the plane. The analyses were carried out using MATLAB software (The MathWorks Inc.).

### Statistical Analysis

Within the 3 risk zones identified in the plot, as described in the previous section, we calculated the cumulative 2-y RI curves to verify the occurrence of statistically significant differences. Cumulative RI curves were generated in R (open-source software, <http://www.r-project.org>) and statistically significant differences were evaluated using the Gray test. All  $P$  values  $\leq 0.05$  were considered significant.

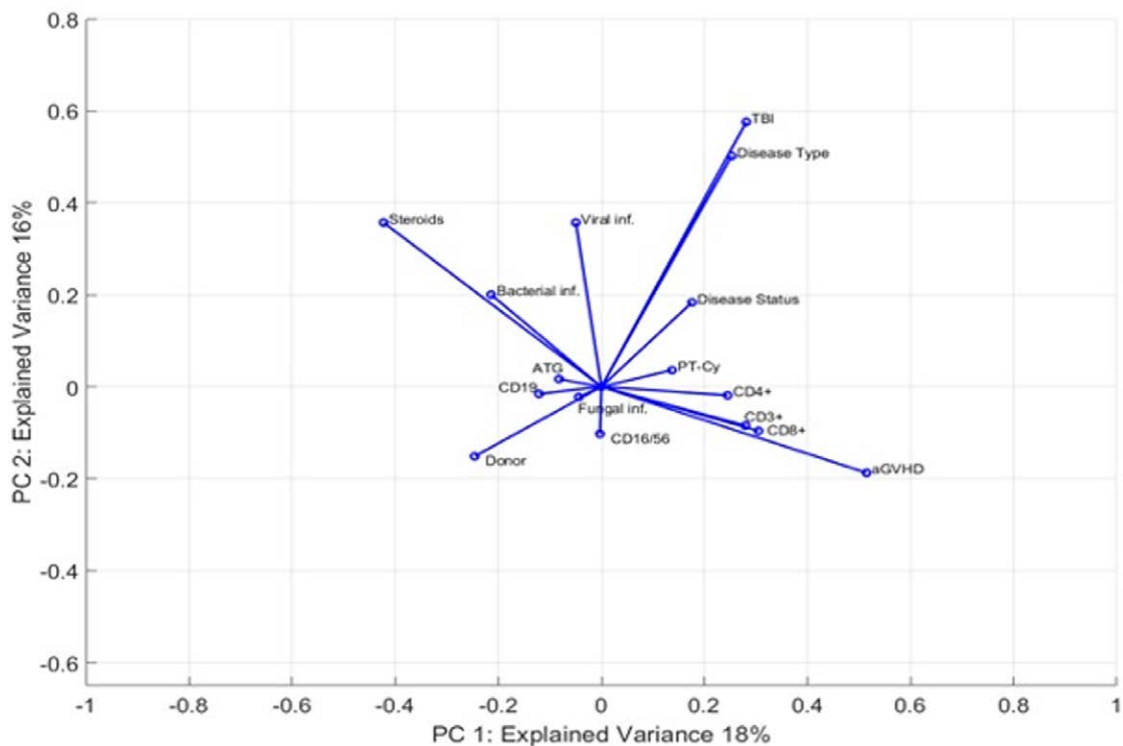
## RESULTS

### Patients

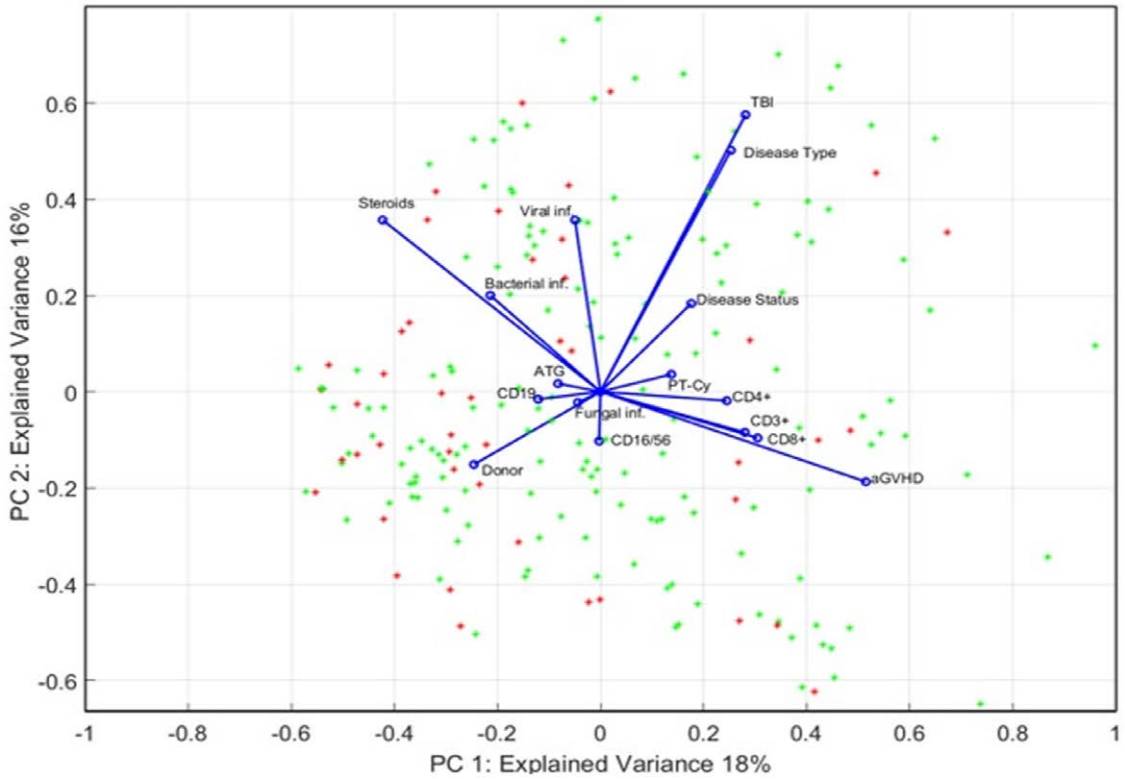
Relapse was observed in 45 (22%) patients. The median time to relapse was 7.4 mo (interquartile range, 6.85). The cumulative incidence of relapse was 22.2% (95% confidence interval [CI], 17.3–27).

### A Multivariate Model of Immune Reconstitution Post-HSCT

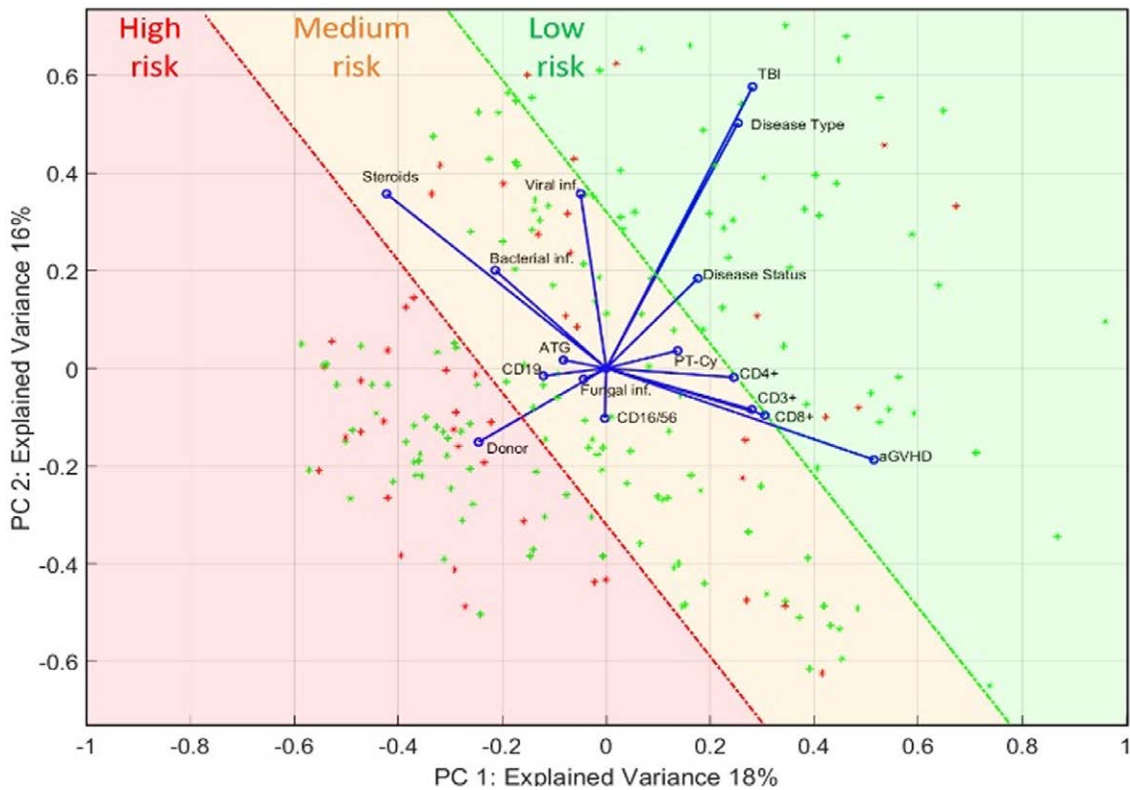
We applied PCA on the data set consisting of 203 patients, of which 16 above-mentioned variables were considered.



**FIGURE 2.** In the plot, each eigenvector represents the contribution of each single variable to the PCs, PC1 and PC2, and it has a length that is directly related to its variance (ie, the more the variance, the more the vector's length, the more the information that is provided by that variable). In other words, it is possible to state that the longer the vector associated with each variable, the more important that variable is to describe the variability in the population. Each vector is projected toward the 2 PCs in the direction that reflects its correlation to the PCs (ie, aGVHD eigenvector is projected toward the PC1, in such a way that reflects its correlation to this latter, because aGVHD eigenvector is projected to the positive values of PC1, the more the variance in aGVHD, the more the contribution of aGVHD to PC1, the more the information provided by aGVHD eigenvector). aGVHD, acute graft-versus-host disease; ATG, antihuman thymocyte globulin; PC, principal component; PCA, principal component analysis; PT-Cy, posttransplantation cyclophosphamide; TBI, total body irradiation.



**FIGURE 3.** Output of the PCA model: patients are reported in a low-dimensional plane defined by the first 2 PCs. Each marker represents a single patient mapped in the low-dimensional model. Red markers stand for relapsed subjects, and green markers stand for nonrelapse. Blue lines (eigenvectors) indicate the contribution to the 2 PCs of each variable included in the model. aGvHD, acute graft-versus-host disease; ATG, antihuman thymocyte globulin; PC, principal component; PCA, principal component analysis; PT-Cy, posttransplantation cyclophosphamide; TBI, total body irradiation.



**FIGURE 4.** Identification of 3 classes of risk as stratified by the PCA model. It is possible to observe a major concentration of relapse cases in the low-left quadrant of the plot (red zone, below the red line). The green line stratifies medium-risk (orange) and low-risk (green) zones and is identified as symmetric to red line with respect to the origin of the plot. aGvHD, acute graft-versus-host disease; ATG, antihuman thymocyte globulin; PC, principal component; PCA, principal component analysis; PT-Cy, posttransplantation cyclophosphamide; TBI, total body irradiation.

We identified a model consisting of the first 2 PCs, which explain 18% and 16% of the total data set variability—of note, these 2 PCs represent the most accurate linear bi-dimensional model of immune reconstitution. Data of the patients were then mapped from the 16-variables' original description to the new 2-dimensional model. In Figure 3, each marker represents a patient in the low-dimensional model. It is evident that patients who experienced relapse (red dots) tend to cluster in the inferior-left quadrant.

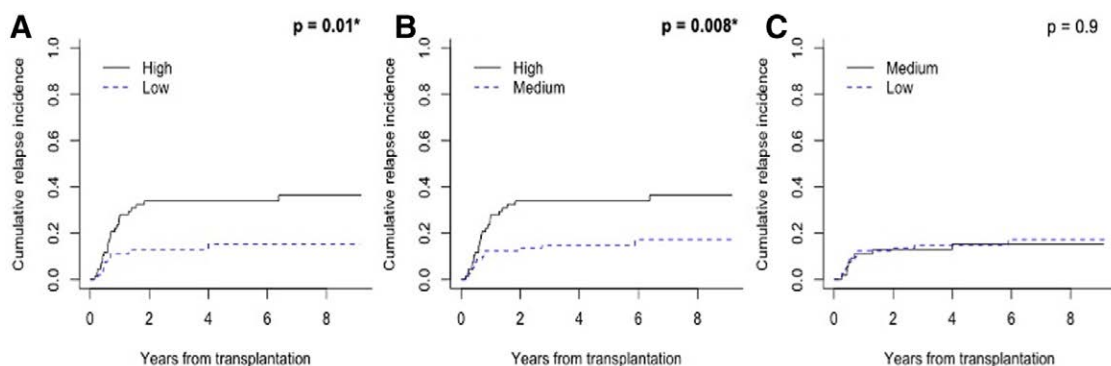
### Identification of Risk Zones and Relapse Incidence

We identified 3 zones in the 2-dimensional plot, supposing a different correlation of each zone with the risk of relapse (see Figure 4). Our hypothesis was that patients who fall in the red region have a higher probability of relapse, which decreases in the orange and green zones. To verify these hypotheses, we calculated the cumulative 2-y RI for each group of patients (high, medium, low risk; see Figure 5). We noted 2-y cumulative incidences of relapse equal to 35.3% (95% CI, 25.6-45), 16.1% (95% CI, 9.2-22.9), and 14.8% (95% CI, 6.7-23) for high-, medium-, and low-risk groups of patients, respectively. We found statistically significant differences between the high-low and high-medium groups ( $P=0.01^*$  and  $0.008^*$ , respectively), whereas no statistical significance was observed in the differences between medium-risk and low-risk groups. Hence, we also calculated the cumulative 2-y RI of the medium-low group, which resulted to be 15.6% (95% CI, 10.1-20.7). Thus, we verified the statistical significance of the difference between the high group and the combination of the medium and low groups (named medium-low,  $P=0.001^*$ ; see Figure 6).

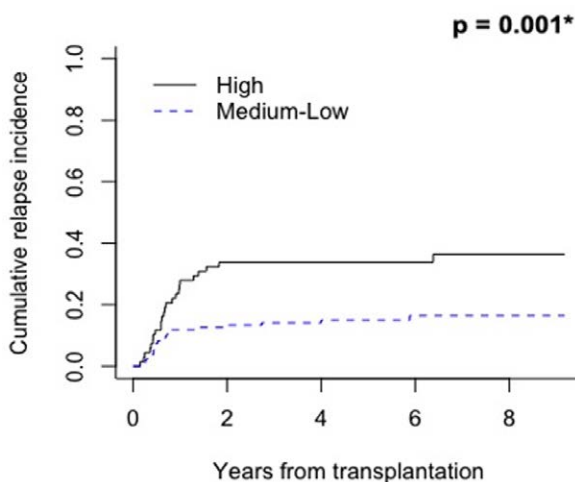
### DISCUSSION

With our study, we were able to elaborate a PCA-based model that represents immune reconstitution and its main influencing factors in a multicentric cohort of pediatric patients who received HSCT for acute leukemias by day 100 posttransplantation. To the best of our knowledge, this is the first study that simultaneously enrolled as input variables of the PCA-model lymphocyte subset numbers - considering age-matched lymphocyte subsets - together with several clinical variables. Further, these latter represent major host's and graft's characteristics notably related to immune reconstitution: disease specificities (status and type of leukemia), conditioning including TBI or chemotherapy alone, GvHD prophylaxis including ATG or in vivo/ex vivo T cell depletion, donor-recipient HLA

matching and early post-transplant steroid therapy as well as bacterial, viral and fungal infections presenting in this same time-frame. Previously, Koenig et al developed a 3-component multivariate model generating a reference domain of ellipsoidal shape on the basis of normal leukocyte subtype counts of healthy children and adolescents. Then, they used this reference domain to classify pediatric patients transplanted for leukemias ( $n=32$ ) as having a superior or inferior chance of becoming long-time survivors and ultimately as having low or high risk of a posttransplant event.<sup>12</sup> Some years later, Mellgren et al<sup>18</sup> developed a PCA model describing patterns of immune reconstitution for different cohorts of patients using as input variables both number of different subsets of T and B lymphocytes and functional tests on B and T cells, but no other clinical variables. They considered both malignant and nonmalignant disorders in 46 pediatric patients and showed dysfunctional reconstitution patterns as predictor of chronic GvHD, relapse, and death. Interestingly, our model extends and improves the previous ones, spreading to relapse prediction. Indeed, we included a considerable number of patients ( $n = 206$ ) affected by acute leukemias and, in addition, we employed as input variables of the PCA model clinical characteristics, so far never included in the mathematical model when this analysis was performed for the same aim.<sup>12,18</sup> Furthermore, in our low-dimensional model, we found that  $CD3^+/CD4^+/CD8^+$  lymphocyte subset numbers at day 100 post-HSCT—with a slightly higher contribution of  $CD8^+$  with respect to the other lymphocytes' subpopulations—and the development of aGvHD had the greatest impact on PC1 (see Figure 3). This is noteworthy because it suggests that a timely and effective immune reconstitution and the development of aGvHD seem to be having the greatest impact in preventing relapse in our cohort of patients. Moreover, our model shows that TBI-based conditioning regimen, disease type and status, negative history of steroid therapy, and the absence of viral infections during the first 100 d (in descending order of importance in our study population, as represented by the different length of the representative eigenvectors; see Figure 3) were related to a better immune reconstitution and ultimately contributed to prevent relapse (see Figure 3). These findings confirm several literature reports that previously analyzed the impact of these isolated variables on immune reconstitution and, ultimately, on transplant outcomes.<sup>6-8,19-23</sup> However, several intrinsic factors may limit these previous studies (such as the reduced number of patients, the heterogeneity of diseases, and other characteristics of the transplantation) and, most importantly, none



**FIGURE 5.** Cumulative 2-y relapse incidence compared between: (A) high- vs low-risk zones, (B) high- vs medium-risk zones, and (C) medium- vs low-risk zones. The Gray test demonstrated statistical significance of the differences between the 2 populations for (A) and (B), whereas no significance is observed for the comparison in (C).



**FIGURE 6.** Cumulative 2-y relapse incidence compared between the high-risk and the medium-low-risk zones. Gray's test demonstrated statistical significance of the differences between the 2 populations.

of them considered the complex interactions among these variables in influencing immune reconstitution.<sup>9,24</sup> From this standpoint, our multivariate PCA model seems to be more reliable<sup>12,18</sup> than previous univariate analyses. Interestingly, donor/recipient HLA-matching had a slight negative impact on both PCs (see Figure 3), suggesting that in this cohort of patients, the more the disparity in the HLA-matching, the more the risk of impaired immune reconstitution and relapse. This appears in contrast with recent literature highlights,<sup>25</sup> but the limited impact of the HLA-matching eigenvector on the 2 PCs in this model and the heterogeneity of the analyzed haploidentical and mismatched unrelated donor transplants (ie, different conditioning regimen, GvHD prophylaxis, both in vivo and ex vivo T-cell depletion) prevent us from drawing any conclusion.

Moreover, taking advantage of the generated PCA model, we were able to ultimately identify 2 statistically significant different groups of patients, characterized by a high and a medium-low risk of 2-y relapse risk ( $P=0.001$ ; see Figure 5) at day 100 post-HSCT. This is noteworthy if we consider that early posttransplantation period is the most critical to enhance the chances of eliminating completely leukemia cells tailoring immunotherapeutic interventions to enhance graft-versus-leukemia reaction. To exploit the possibility of timely identifying high-risk patients, either donor/recipient chimerism<sup>26-28</sup> or pre-<sup>29-34</sup> and post-HSCT<sup>33-38</sup> minimal residual disease (MRD) measurement has been investigated in the past years, yielding interesting results. Recently, a risk score based on MRD assessment in peri-HSCT period has been validated in pediatric patients affected by ALL,<sup>39</sup> but similar results are not available for children affected by AML yet. In this context, our model includes either pediatric patients affected by ALL, AML, and myelodysplastic syndrome and it could be considered as an easy-to-use complementary tool with regard to existing solutions<sup>39</sup> to define patients who could benefit of specific therapeutic approaches. Indeed, immunosuppression tapering or withdrawal,<sup>26,40,41</sup> donor lymphocyte infusion,<sup>42</sup> or cytokine-induced killer cells<sup>43</sup> could be more effectively targeted using this approach. Moreover, more recent leukemia-specific treatments, such as monoclonal antibodies,<sup>44</sup> bispecific T-cell engager therapy,<sup>45</sup> or chimeric antigen receptor-modified T cells,<sup>46</sup> could be studied in high-risk patients defined applying our tool.

It is important to mention that our study has some limitations. First of all is its retrospective design. Furthermore, we carried out our analyses on a cohort of subjects considered as a test set, but a validation set is required to verify whether our model of immune reconstitution could be used as a predictive tool in clinical setting. In addition, we acknowledge the relevance of peri-HSCT MRD measurement for relapse prediction, and future developments of this work, in a prospective study, will investigate the inclusion of this variable, trying to improve patients' stratification and allocation to the different risk scores. Finally, we did not consider functional analysis on T and B cells, as we pursued the aim to generate a tool based on clinical and easily available variables to increase its routine applicability.

The results of our pilot study indicate that our PCA-based model could accurately describe the complex interactions between immune reconstitution and its influencing factors in a large cohort of pediatric patients affected by acute leukemias. Moreover, our model presented here also serves as a reliable risk-defining model for relapse. If validated, it could definitely serve as a predictive tool and could be taken into account for clinical trials or for individualized patient counseling.

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