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ANALISI DELLE MICROVESCICOLE CIRCOLANTI IN PAZIENTI SOTTOPOSTI A TRAPIANTO ALLOGENICO DI MIDOLLO E CORRELAZIONE CON L'INSORGENZA DI GVHD

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Abstract

Acute and chronic Graft Versus Host Disease (GVHD) are the major complications of allogeneic hematopoietic stem cell transplantation (HSCT). Predictors of GVHD are rearly routinely implemented and the majority refers to the interaction between donor immune system and the recipient immune/inflammatory make-up in the post-transplant phase. With the aim to investigate the role of the recipient's immune/inflammatory status before stem cell transplantation, we analyzed the circulating extracellular vehicles (EVs) in plasma and serum of HSCT patients. In an explorative cohort of 39 patients, we observed high levels of CD69+EVs in GVHD patients at pre-HSCT infusion time point up to day +90. Recent studies have highlighted the role of recipient-derived, tissue-resident memory T (TRM-T) cells, a long-lived, pro-inflammatory population known to persist despite conditioning regimens, in the initiation and propagation of GVHD. To support the hypothesis that CD69+EVs originate from TRM-T cells, we assessed the presence of CD103 protein, a hallmark of TRM-T cells, on the CD69+ EVs. The results showed that double-positive CD69+CD103+ EVs were present at higher levels in GVHD patients' serum. Furthermore, the study found a strong negative correlation between serum Sphingosine 1-Phosphate Receptor (S1PR) and CD69+EVs levels, a finding consistent with literature data showing that CD69 expression impairs the S1P and S1PR axis function, which halts the egress of TRM-T cells from tissues. In a validation cohort of 100 patients, multi flow cytometry and multicolor diffractometric analysis, found that day -6 and day -1 before HSCT serum level of CD69+ EVs were predictive of acute and chronic GVHD. Our data show that pre-transplant CD69+EVs are likely to be the telltale of pre-transplant TRM burden and are strong predictors of GVHD in HSCT patients.

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Chapter 1 – Introduction

1.1 aGVHD markers

Acute Graft Versus Host Disease (aGVHD) is the most frequent complication after disease relapse in patients undergoing allogeneic bone marrow transplantation. Its incidence reaches 30-50% of patients undergoing transplantation [1]. The manifestations of this complication mainly involve 3 body districts, the skin, where it causes a generally morbilliform rash whose severity depends on its extent, the liver, where it causes a generally cholestatic hepatitis, and the intestine, where it causes a protein-losing diarrhea [2].

The diagnosis of aGVHD, although confirmation through histological analysis of samples from the affected systems is possible, is often clinical based on the characteristic presentation and the exclusion of any alternative causes (usually infectious) of the ongoing manifestations.

The standard therapy for aGVHD consists of high-dose corticosteroids, even if the observed response is suboptimal, especially in the more severe forms, i.e., with the involvement of gastrointestinal system. Generally, the response to corticosteroids is observed in about 50% of patients. Subsequent lines of therapy consist of various immunosuppressive drugs, and although research in this area is growing and has led to the recent approval of second-line drugs, the outcome for patient's refractory to corticosteroid therapy is still unsatisfactory and the mortality in these cases is still high.

The development of biomarkers in this context is of particular interest. Finding a reliable biomarker for aGVHD would be useful from multiple points of view. First, a marker capable of predicting the onset of aGVHD or identifying patients at higher risk would allow for the development of better prophylaxis strategies and for weighing immunosuppression based on risk. Furthermore, an early diagnosis, before the onset of more severe manifestations, would allow for the evaluation of pre-emptive therapy strategies. In addition, an effective aGVHD marker could allow for a more precise evaluation of the degree of response to therapies, which is currently based on the clinical assessment of the transplant physician. This would also make it possible to design clinical trials for new drugs with more solid endpoints.

The most studied biomarkers in aGVHD are those included in the MAGIC algorithm. Initially a pioneeristic work assessed 120 plasma protein in 42 allotransplant recipients, including immunoregulatory cytokines, cytokines that act as growth factors, soluble cytokine receptors, various protease sand protease inhibitors, extracellular stromal molecules and mediators involved in the blood coagulation and complement cascade. They found 8 soluble proteins with a significant different level between patients who developed aGVHD and those that didn't. Those 8 proteins were further tested in a training cohort (n=242) and a validation cohort (n=142) identifying a panel of 4 proteins: IL-2Ra, TNFR1, IL-8, and hepatocyte growth factor (HGF), obtaining an AUC of the ROC curves of 0.91 and 0.86 respectively. Furthermore, the panel can predict the survival of patients with GVHD independently of the severity grade [3].

Elafin a protease inhibitor mainly located in keratinocytes and is induced during inflammation was tested in addition to the panel of IL-2Ra, TNFR1, IL-8, and HGF, in. a cohort of 111 patients and was proved as the most effective markers in discriminating patients with aGVHD, moreover the addiction of elafin improved the performance of the biomarkers panel [4].

Regenerating islet-derived 3α (REG3α) in a protein produced by Paneth cells in the intestine and acts downstream to the IL-22 pathway to induce intestinal barrier repair. RAG3a plasma levels were measured in a prospective cohort of 112 patients at day of aGVHD diagnosis, after 14 days and after 28 days along with IL-2Ra, TNFR1, IL-8, HGF and elafin. Day 0 biomarker panel independently predicted mortality by day 180 from diagnosis, moreover biomarker panel measured at day 14 and day 28 predicted day 28 response status and day 180 survival status [5].

The most studied biomarker in aGVHD is ST2, discovered by analyzing samples from aGVHD patients resistant to therapy [6]. The marker was discovered by a proteomic screening of 571 plasma proteins. Between those only 6 showed a meaningful increase in aGVHD treatment resistant patients and were further assessed in a cohort of 381 patients and confirmed in three other cohorts for a total 673 patients. The 6 plasma protein included ST2, RAG3α , macrophage inhibitory factor, IL1 receptor 2, lipocalin 2,lymphatic-vessel endothelial hyaluronan receptor 1. ST2, the soluble receptor for the alarmin IL-33, which is shed from multiple cell types in the injured gastrointestinal crypt, outperformed the six-biomarker panel in predicting overall 6-month mortality across patient cohorts. However, for patients with lower GI aGVHD, combining ST2

and REG3α improved risk stratification accuracy compared to either biomarker alone [6].

This marker has been validated in many types of transplants, such as cord blood transplantation [7], transplants with non-myeloablative conditioning [8], and T-replete haploidentical transplantation [9]. The level of ST2 on days +7 and +14 post-transplant has also been validated as a prognostic marker for GVHD in large prospective cohorts [10,11].

Some fecal proteins such as calprotectin and alpha-1-antitrypsin are possible biomarker candidates. These proteins have been correlated with the response to corticosteroids in patients with intestinal aGVHD [12]. The association between these markers and intestinal aGVHD has been proposed in many studies, however, they were all small, single-center studies; therefore, a definitive validation is still pending.

The Mount Sinai consortium developed the MAGIC algorithm including ST2 and REG3α. In a first study the consortium measured 4 biomarkers (ST2, REG3α, TNFR1, and IL-2Rα) at day 7 after transplantation in order to model 6-month NRM to identify the best algorithm that defined 2 distinct risk groups. A model including ST2 and REG3α was able to identify patients at higher risk of NRM at 6 months and was validated in a different test set and in a validation multicentric cohort [13].

The MAGIC algorithm was tested even in patients developing aGVHD after 1 week of systemic therapy. Steroid resistant patients could be separated into two group with strikingly different OS and NRM in three independent cohorts. Moreover, MAGIC algorithm measured at aGVHD onset was predictive of NRM along with steroid refractoriness and GVHD severity (according to Minnesota score) but MAGIC algorithm showed a significant better ROC curve AUC [14].

1.2 cGVHD markers

Chronic graft-versus-host disease (cGVHD) is one of the main causes of morbidity and long-term mortality after allo-HSCT, occurring in about 30-50% of patients.

The diagnosis of cGVHD, according to the National Institutes of Health (NIH) consensus criteria, can be made if there is presence of a diagnostic feature, or if there is at least one distinctive manifestation in addition to radiologic, histologic or laboratory evidence of GvHD from any site [15]. Chronic GvHD can potentially involve any organ

system. Organ involvement is more heterogeneous and disease manifestations are more variable in cGVHD compared to aGVHD.

Although some novel therapeutic approaches have shown a good efficacy in cGVHD therapy, it is unlikely that they will completely overcome drug resistance, so combined therapies could be promising in the next generation of trials. The value of potential cGVHD biomarkers is in their usefulness for prognosis, predicting therapeutic responses, and for identifying new therapeutic targets. Identification of cGVHD biomarkers requires specific consideration of the sensitivity and specificity in subgroups with different clinical characteristics. Any biomarker should be carefully evaluated during the verification phase.

Numerous biomarkers have been proposed although none has moved to clinical application due to lack of true suitable candidates that have been evaluated in large prospective cohorts. Nevertheless, the research in this direction is active and numerous candidates have been proposed.

Donor B cells contribute substantially to the development of cGVHD, the first insight in of the pathogenetic involvement of B-cells in cGVHD comes from the observation that an altered B cell homeostasis and excess of BAFF in patients developing cGVHD[16]. Moreover, CD19+ CD21− B cells have been reported correlating with disease severity and organ manifestations [17]. In a prospective study including 136 patients (46 BOS, 41 no cGVHD, 49 cutaneous cGVHD) patients with newly diagnosed BOS had significantly higher percentages of CD19+ CD21low B cells, BAFF levels, and BAFF/CD19+ ratios compared with patients without cGVHD [18]. In the same way a study comparing serial measurement from day + 100 in 163 patients with cGVHD and 64 without cGVHD found elevated frequencies of CD19+ CD21- B cells significantly correlated with first diagnosis of cGVHD. CD19+ CD21- B cells on day +100 were analogously associated with later development of cGVHD and could serve as a prognostic biomarker for clinically significant cGVHD [19].

Beside B cells other cellular population like T reg [20], B reg [21], Th 17 [22], NK cells [23]have been linked to cGVHD development but very few cellular biomarkers have been identified from clinical studies, including discovery and independent verification as requested by the NIH consensus development group [24].

1.3 Extracellular vesicles as GVHD biomarkers

Extracellular vesicles (EVs) are particles coated with a phospholipid membrane actively secreted by cells in different ways and which contain various biological molecules, including lipids, nucleic acids (DNA, RNA, miRNA) and proteins, as well as presenting membrane proteins. The composition reflects the cell of origin in addition to its physiological or pathological state. Thanks to these characteristics, extracellular vesicles have been used as biological markers in many medical fields such as cardiology, neurology, oncology and onco-hematology [25].

The analysis of extracellular vesicles in the field of GVHD is currently still little explored and the field is more active in the aGVHD context. Extracellular vesicles could be characterized studying their surface molecules expression or their contents.

PDL1+EVs accumulation have been linked to the development of aGVHD in a cohort of 39 patients. Moreover, PDL1+EVs levels correlated positively with GvHD grade and declined (only) on successful therapeutic intervention suggesting the hypothesis that is possible to use PDL1+EVs to monitor aGVHD [26].

In another study on 41 patients with multiple myeloma who received allo-transplant the authors used 13 antibodies against specific membrane proteins to determine the EVs levels expressing the corresponding antibodies. The CD146, CD31, and CD140-α+EVs had a strong correlation with the appearance of aGVHD. Among them, CD146 (MCAM-1) increased the risk of aGVHD by nearly 60%, whereas CD31 (PECAM-1) and CD140 (PDGFR) decreased the risk of almost 40% and 60%, respectively [27]. In a follow-up study they further measured the expression profiles of CD146, CD31, CD140a, CD120a, CD26, CD144, CD30, and their miRNA loading on EVs in 32 consecutive patients. They found that the expression profile of EVs and plasma biomarkers (such as ST2, sTNFR1, and REG3a) potentially correlated with aGVHD and the combination of CD146, sTNFR1, and miR-100 or miR-194 in EVs has diagnostic efficacy, indicated by an ROC curve AUC of > 0.975, suggested that the expression profile of EVs was expected to be a promising biomarker for the early diagnosis of aGVHD [28].

The nucleic acid EVs content have been investigated too as a potentially associated to aGVHD pathogenesis and thus suitable as biomarker.

In a study involving 20 consecutive patients an acoustic fluidic capture technology has been used to capture EVs in plasma samples 12 weeks pre- and post-allo-HSCT. Through the analysis of the miRNA expression profiling of EVs, the expression of miR-15b-3p, miR-30a-5p, miR-130a-3p, miR-145-5p, and miR-342-3p in plasma EVs was conspicuously augmented after transplantation; in contrast, the expression of miR-18a-5p, miR-92b-3p, miR-93-5p, miR-141-3p, and miR-486-5p was significantly diminished. Importantly, they found that upregulated miRNAs in plasma EVs were positively correlated with infection and GvHD, respectively, while the downregulated miRNAs were negatively correlated with these complications.

Although these results are encouraging, further studies are needed to characterize and define extracellular vesicles as reliable markers for aGVHD.

In cGVHD the potential of EVs as biomarker is not has been explored yet but, considering that the majority of the pathogenetic events that brings to cGVHD take place in the peripheral tissues, EVs are potentially an interesting tool, being able to act as a detector of cellular events not explorable through the analysis of cellular populations in peripheral blood.

1.4 Study rational

Despite significant advances in understanding GVHD pathogenesis, the identification of reliable and clinically applicable biomarkers to predict GVHD risk remains an open issue [14,29–31]. Recent studies have highlighted the role of recipient-derived, tissue-resident memory T (TRM-T) cells a long-lived, pro-inflammatory population known to persist despite conditioning regimens in the initiation and propagation of GVHD [32–35]. TRM-T cells are retained in tissues by surface markers such as CD69 and CD103, which inhibit their egress into circulation [36–39]. One of the main challenges in verifying the activation and involvement of such tissue-resident cells is the hindrance of assessing their presence and activation status without resorting to invasive histological methods. A promising alternative lies in the use of extracellular vehicles (EVs), which are lipid-bound particles released by cells and capable of carrying surface markers representative of their cell of origin [40]. EVs derived from TRM-T cells may thus serve as accessible, blood-based biomarkers of otherwise undetectable immune populations. In our previous work, we reported that serum $CD69^{+}/CD103^{+}$ EVs can serve as surrogate

markers of TRM-T cells during the pre-transplant conditioning phase (days -6 to -1) in patients undergoing allogeneic HSCT [30]. Building upon this observation, we conducted a comprehensive analysis of EVs profiles in an exploratory cohort of 39 HSCT patients sampled before and after transplantation (up to day +90). We then extended our investigation to an independent pre-transplant cohort (expansion cohort n=100) to evaluate the predictive value of $CD69^{+}$ EVs for GVHD risk. In addition, we characterized the surface phenotype of EVs to confirm their exosomal origin and examined their biological effects on myeloid cells to gain mechanistic insights into their potential immunomodulatory roles.

Chapter 2 - Methods

2.1 Patient enrolment and sampling strategies

We present a study conducted at IRCCS AOU S. Orsola-Malpighi of Bologna focusing on the relation between circulating extracellular vesicles and hematopoietic stem cell transplantation (HSCT) outcomes with particular interest in acute and chronic GVHD. The study involved an overall population of 139 patients transplanted between 2018 and 2022. Inclusion criteria were age (>17 years), any type of allogeneic HSCTs and written informed consent.

All patients were divided into two cohorts: group 1 (39 patients, explorative cohort) and group 2 (100 patients, expansion cohort)

The exploratory cohort underwent a peripheral blood sampling at the following time points: day -6 before HSCT (which was before conditioning), day -1 before HSCT, day +30 and day + 90 after HSCT.

The expansion cohort underwent a peripheral blood sampling at day -6 before HSCT (which was before conditioning) and at day -1 before HSCT.

All patients received a three-drug prophylaxis for transplant disease. Patients who underwent a haploidentical family donor transplant received a prophylaxis containing post-transplant cyclophosphamide associated with tacrolimus and mycophenolate mofetil as previously described[41,42]. Patients who underwent a transplant from a compatible family donor or a registry donor were given a prophylaxis comprising low doses of ATLG (Grafalon, Neovii) associated with a calcineurin inhibitor (cyclosporine or tacrolimus) and short-term methotrexate or mycophenolate as previously described[43].

All patients were administered anti-infective prophylaxis with levofloxacin and posaconazole during the cytopenia period and with acyclovir for up to 24 months after the transplant. In the event of positivity for Cytomegalovirus (CMV) -DNA in the blood, pre-emptive therapy was administered using ganciclovir or foscarnet when the CMV-DNA exceeded 10,000 copies/ml in whole blood. EBV monitoring was performed twice a week during the first 100 days after the transplant, then twice a month until the sixth month after the transplant. Prophylaxis with trimethoprim-

sulfamethoxazole was administered three times a week from engraftment until twelve months after the transplant. All patients received filtered and irradiated blood products. Acute GVHD was diagnosed and graded according to MAGIC criteria[2], while chronic GVHD was diagnosed and graded in accordance with NIH guidelines[15].
All patients were enrolled in a monocentric prospective tissue study (MET_SCT_2018; 151/2018/Sper/AOUBO; NCT 03871269). The study was conducted in compliance to Helsinki Declaration and all patients enrolled in the study provided a written informed consent.

2.2 Samples processing and stocking

Explorative cohort patients' blood samples were collected in 7 ml tubes containing EDTA as an anticoagulant, while expansion cohort patients' blood samples were collected in 7 ml serum separating tubes. The tubes were centrifuged at 1500 g for 15 minutes and the supernatant was divided into 1 ml aliquots stored in cryovials at -80°C until the time of analysis.

2.3 MACSPlex exosome analysis

Plasma samples collected from patients from exploratory cohort at each time point were analyzed by MFC using MACSPlex exosome kit (cod# 130-108-813) from Miltenyi biotech (Germany). The samples were processed as follows: 50 uL of plasma were diluted to a final volume of 120 uL with MACSPlex buffer (MPB) and incubated overnight protected from light in an orbital mixer (800 rpm at 10°C) together with the MACSPlex Exosome Capture Beads consisting of 37 groups of beads, each group coated with a specific antibody against a determined epitope (namely CD3, CD4, CD19, CD8, HLA-DRDPDQ, CD56, CD105, CD2, CD1c, CD25, CD49e, ROR1, CD209, CD9, SSEA-4, HLA-ABC, CD63, CD40, CD62P, CD11c, CD81, MCSP, CD146, CD41b, CD42a, CD24, CD86, CD44, CD326, CD133/1, CD29, CD69, CD142, CD45, CD31, CD20, CD14). The MPB without added sample was used as a control. The beads were then washed with 1 mL of MPB and centrifuged at 3000 g for 15 min at 10°C. 1 mL of supernatant was carefully aspirated and 15 uL of MACSPlex Exosome Detection Reagent were added (5 uL for each anti-CD9, anti-CD63, and anti-CD81 antibody

conjugated with the APC fluorochrome). The resulting sample was incubated for 1 hour protected from light in an orbital mixer (450 rpm at 10°C). After a further wash like the previous one, the sample was incubated for 1 hour protected from light in an orbital mixer (450 rpm at 10°C). Then 1 mL was aspirated, leaving 150 uL in the tube. The samples were resuspended and 100-120 uL were used for analysis.
Median fluorescence intensity (MFI) was measured on a MACSQuant-Analyzer-10 flow cytometer (Miltenyi) according to previous validation studies [44,45]. All markers were analyzed simultaneously. Surface epitope levels were referenced to EV-specific epitopes by subtracting the respective fluorescence values of blank control from MFI values for individual surface epitopes, and by normalizing them for CD9/CD63/CD81 MFI, reflecting EV concentration.

2.4 ExoView™ analysis

Serum samples from 17 patients of the expansion cohort were analyzed by ExoView™ platform (Leprechaun instrument_Unchained Laboratories, CA, USA), following the manufacturer's protocol. We analyzed the presence of $CD69^+$EVs and $CD103^+$EVs by ExoView which is designed for the CHIP-based immuno-capturing of EVs via established EVs/exosomal markers (CD9, CD63, CD81) and the subsequent assessment of EVs via fluorochrome-conjugated antibodies (**Supplemental Figure 1A**) [46]. The detection antibodies used were anti-CD69-APC_H7 clone FN50 (BD Biosciences), anti-CD103-AlexaFluor 488 clone BerAct8 (Biolegend, San Diego, CA, USA). The analysis was performed according to the manufacturer's instructions. Chips analysis was performed using the ExoView™ R200 reader endowed with ExoView Scanner software (v 3.0). The total number of $CD69^+$EVs, $CD103^+$EVs and the double positive $CD69^+$/$CD103^+$EVs were calculated as the sum of EVs captured by anti-CD9, anti-CD63, and anti-CD81 on the chip surface.

2.5 Dual Stochastic Optical Reconstruction Microscopy (STORM) analysis

Plasma $CD69^+$EVs were labeled with anti-CD69 Alexa 647-conjugated human recombinant (clone FN50, cod# MA5-18150; Thermofischer, USA) and CF680 conjugated rabbit recombinant CD63 (clone EPR21151 cod#ab217345 Ab Idiotype;

Abcam) and imaged in Spectral demixing dSTORM (laser power 640: 300 mW, 405: uniform gradient from 0 to 40 mW during the entire acquisition, dichroic mirror T700 for 10000 frames/acquisition over a constant HiLo-plane angle and 50 ms of exposure time). The acquired single molecule blinking videos were super-localized with the NEO_analysis software (Abbelight, France), to obtain super-resolution coordinate tables. Channel alignment, spectral demixing was performed in NEO_analysis (Abbelight). Single-EV segmentation was performed using DBSCAN in NEO_analysis (Abbelight). EVs sizing and color assignment were performed with custom Python scripts and data are available in the **Supplemental methods** and **supplemental tables** (see below).

2.6 Multiparametric flow cytometry (MFC) analysis of plasma and sorted CD69^{+}EVs

Cytoflex SRT equipment (Beckman Coulter, Brea, California, USA) was used to sort CD69^{+}EVs. Side scatter (SSC)-Violet laser were combined for EVs gating strategy. Sorting regions were defined by fluorescent markers and 10^6 events were obtained in the collection tube (**Supplemental Figure 1B**). Sorted CD69^{+}EVs were analyzed with an ad-hoc custom antibody mix (CD45-FITC, CD4-APC, CD62L-PE, CD45RA-PE-CY7, CD56-BV421 cod# 626682, provided by Cocktail Manufacturing BD Custom Technology Team, BD Biosciences, USA).

Serum level and immunophenotype of EVs were analyzed by MFC employing BD FACSLyric (Becton Dickinson, Franklin Lakes, New Jersey, USA) as previously described [30,47]. Briefly, 5 µl of patients' serum was added to 95 µl of filtered PBS and 95 µl of a mix of fluorochrome conjugated moAbs namely CD31 PE-Cy-5.5, clone WM-59 (BD Biosciences Cat# 563651), CD45 BV510, clone HI30 (BD Biosciences Cat# 563204), CD41a PE, clone HIP8 (BD Biosciences Cat# 557297) and anti-CD69-APC_H7 clone FN50 (BD Biosciences Cat#560737). FITC-conjugated phalloidin and APC-conjugated Lipophilic Cationic Dye (BD Biosciences) were used to exclude non-EVs events from the analysis. The number of CD69+EVs/ml was assessed using the TruCount BD system (BD Biosciences)

2.7 EVs count and size: Nanoparticle Tracking Analysis (NTA)

The EVs size and concentration were determined by NTA using the NanoSight RS300 system (Malvern Panalytical, Malvern, United Kingdom). Each sample was diluted (1:100) in PBS (filtered with a 0.1 μm filter). For each sample, a syringe pump flow of 30 was applied. Three videos of 60s each were recorded and analyzed, calculating an average number of EV size and concentration (particles/ml). All samples were characterized with NTA 3.2 analytical software.

2.8 Protein ELLA and Elisa test

Serum levels of cytokines (Interleukin-6, Interleukin-10, Interferon-gamma, beta and alpha, Granzyme B, Interleukin-15, CXCL9/MIG and Neurofilament light protein, NFL) were assessed by Protein ELLA system (Biotechne, San Josè California) at day -1 before transplant. Serum level of Sphingosine 1-phosphate receptor and sphingosine 1-phosphate were assessed by Elisa test (cat# MBS3803384 and cat# MBS7254488 respectively, MyBiosource San Diego, California, USA). ST-2/IL-33 R Immunoassay (cat#: DST200 Biotechne, San Josè California) was employed to assess serum of HSCT patients.

2.9 EVs purification by Size Exclusion Chromatography (SEC) and Extracellular vesicles Dot Blot analysis

SEC separation was performed using Pure EVs column system (Hansa_Biomed, Estonia). 30ul of EVs were spotted onto a nitrocellulose membrane (Bio-Rad, CA, USA). Primary antibodies used were: CD69 (1:1000 dilution; clone FN50, cod#130-108-065 Miltenyi Biotech), CD63 (1:1000 dilution, clone TS63; Thermo Scientific, Italy), and APOA4 (1:2000 dilution, clone ab59036; Abcam, Cambridge, UK). Immune complexes were visualized using the Clarity solution (Bio-Rad).

2.10 Cell Cultures

THP1 and MOLM-13 cell lines were grown in RPMI supplemented with 10% FBS, 1% of penicillin/streptomycin and 1% of Glutamine in a 5% CO2 incubator. 50,000 cells were seeded and exposed to 1,000 EVs/cell for 24h and lysed with Qiazol (Qiagen, Germany).

2.11 RNA-seq analysis

RNA-seq assay was performed on THP1 and MOLM-13 cells exposed to EVs collected from patients with or without GVHD. RNeasy Mini Kit (Qiagen, Germany) was used to extract RNA from THP1 and MOLM-13 cell lines. The yield of RNA was assessed with NanoDropOne (Thermo Fisher Scientific, USA). RNA samples were purified using RNAClean XP beads (Beckman Coulter, Brea, CA, USA). Libraries were pooled and stored at -20°C until sequencing on NovaSeq 6000 Illumina system (Illumina, San Diego, CA, USA). Standard RNA-seq pipeline on Illumina DRAGEN Bio-IT Platform 3.9 was used for transcriptomic data analysis. Normalized data were analyzed using GeneSpring GX software (Agilent Technologies, CA, USA) to identify differentially expressed genes (moderated t-test with FDR 5%) in EVs vs. control and GVHD yes vs. no groups.

2.12 Statistics, bioinformatics and diagnostic modeling

Scalar variables were analyzed using the Kolmogorov-Smirnov test to determine their distribution. Variables with a normal distribution were described using mean ± standard deviation; variables with a non-normal distribution were described using median and interquartile range. Analysis of variance with Bonferroni's post-hoc test and Kruskal-Wallis's test were used to compare variables with normal and non-normal distributions, respectively. Categorical variables were compared using the chi-square test. Correlations were evaluated using Pearson's test. ExoView measured EVs and MFC $CD69^{+}$EVs were analyzed with Mann-Whitney test and Kruskal-Wallis Dunn test with benjamini-hochberg correction. Cumulative incidence (CI) analysis was used to estimate the probability of aGVHD, aGVHD 2-4, cGVHD, cGVHD moderate-severe, treatment related mortality (TRM). Death and relapse without GVHD were used as competitive events for the cumulative incidence analysis of aGVHD, aGVHD 2-4, cGVHD and cGVHD moderate-severe, while relapse were used as competing events for TRM. Optimal cut-off for $CD69^{+}$EVs was determined by Receiving Operating

Curve (ROC) analysis as the value that maximizes Youden's Index for every outcome (aGVHD, aGVHD 2-4. cGVHD and cGVHD moderate-severe). Gray's test was used to compare the CI of each outcome between patients with a level of $CD69^{+}$EVs above and below the optimal cut-off. The Fine-Gray competing events model was used to evaluate the association of the covariates with the outcomes. Covariates having a p-value <0.05 for association the outcomes in the univariate analyses were added to a Fine-Gray competing event regression model using stepwise backward selection based on p-value to determine covariate association with each outcome. The Kaplan-Meier method was used to estimate the probability of Graft Free Relapse Free Survival (GFRFS) and Overall Survival (OS). To determine the optimal cut-off, we used maximally selected rank statistics, while log-rank test was used to compare GFRFS between groups. Covariates having a $p<0.05$ according to Cox regression model were added to a multivariable model applying stepwise backward selection based on p-value to determine covariate associations with the outcome. The statistical analysis was performed using the software IBM SPSS Statistics 22 (IBM), Python 3.5 (library, scikit-learn), GraphPad PRISM 9.0a, RStudio version 2023.06.0+421 (2023.06.0+421).

Chapter 3 - Results

3.1 Patients characteristics and overall outcomes

A total of 139 patients, 84 males and 55 females with a median age of 55 years (range 18–71) were included in this analysis. The first 39 patients belong to the exploratory cohort: while the following 100 patients, to the expansion cohort. Characteristics of patients of overall population and of both cohorts are displayed in ***Table 1*** and in ***Figure 1-3***.

In the whole population, median follow-up was 755 days (95% CI, 728-897). Overall survival (OS) at 1 year was 86.8% (95% CI, 81.3-92.7) while TRM at 1 year was 6.5% (95% CI, 3.2-11.5). Overall, 62 (45%) patients in the whole population developed aGVHD of which 42 (30%) developed aGVHD II-IV and 20 (14%) aGVHD III-IV. cGVHD was diagnosed in 34 (24%) of which 29 (21%) patients developed cGVHD moderate-severe. aGVHD CI at days 100 in the whole population was 36.7% (95% CI, 28.7-44.7), while aGVHD grade II-IV and grade III-IV was 24.5% (95% CI, 17.7-31.9) and 10.8% (95% CI, 6.32-16.6), respectively. cGVHD incidence at 1 year was 21.8% (95% CI, 15.3-29.0) and cGVHD moderate-severe incidence at 1 year was 18.2% (95% CI, 12.2-25.1).

In the explorative cohort, with a median follow up of 1463 days (95% CI, 1313-1643) the aGVHD CI at 100 days was 28.2% (95% CI, 15.1-42.9), incidence of aGVHD grade II-IV and grade III-IV was 20.5% (95% CI, 9.5-34.4) and 10.3% (95% CI, 3.2-22.2), respectively. Then, 12.8% (95% CI, 4.6-25.4) of patients developed cGVHD at 1 year from HSCT. OS at 1 year was 92.3% (95% CI, 84.3-100.0), while TRM was 5.1% (95% CI, 0.9-15.4) at 1 year.

In the expansion cohort, median follow-up was 656 days (95% CI, 595-731). In this former cohort 49 (49%) patients developed aGVHD, 32 of which reached grade II or higher. aGVHD CI at 100 days was 40.0% (95% CI, 30.3-49.5), while at the same time point, aGVHD II-IV CI and aGVHD III-IV CI were respectively 26.0% (95% CI, 17.8-34.9) and 11.0% (95% CI, 5.8-18.0). cGVHD CI at 1 year was 25.4% (95% CI, 17.2-34.3). OS and TRM were 84.6% (95% CI, 77.7-92.1) and 7.1% (95% CI, 3.1-13.3) at 1 year.

Table 1 *Patients characteristics*

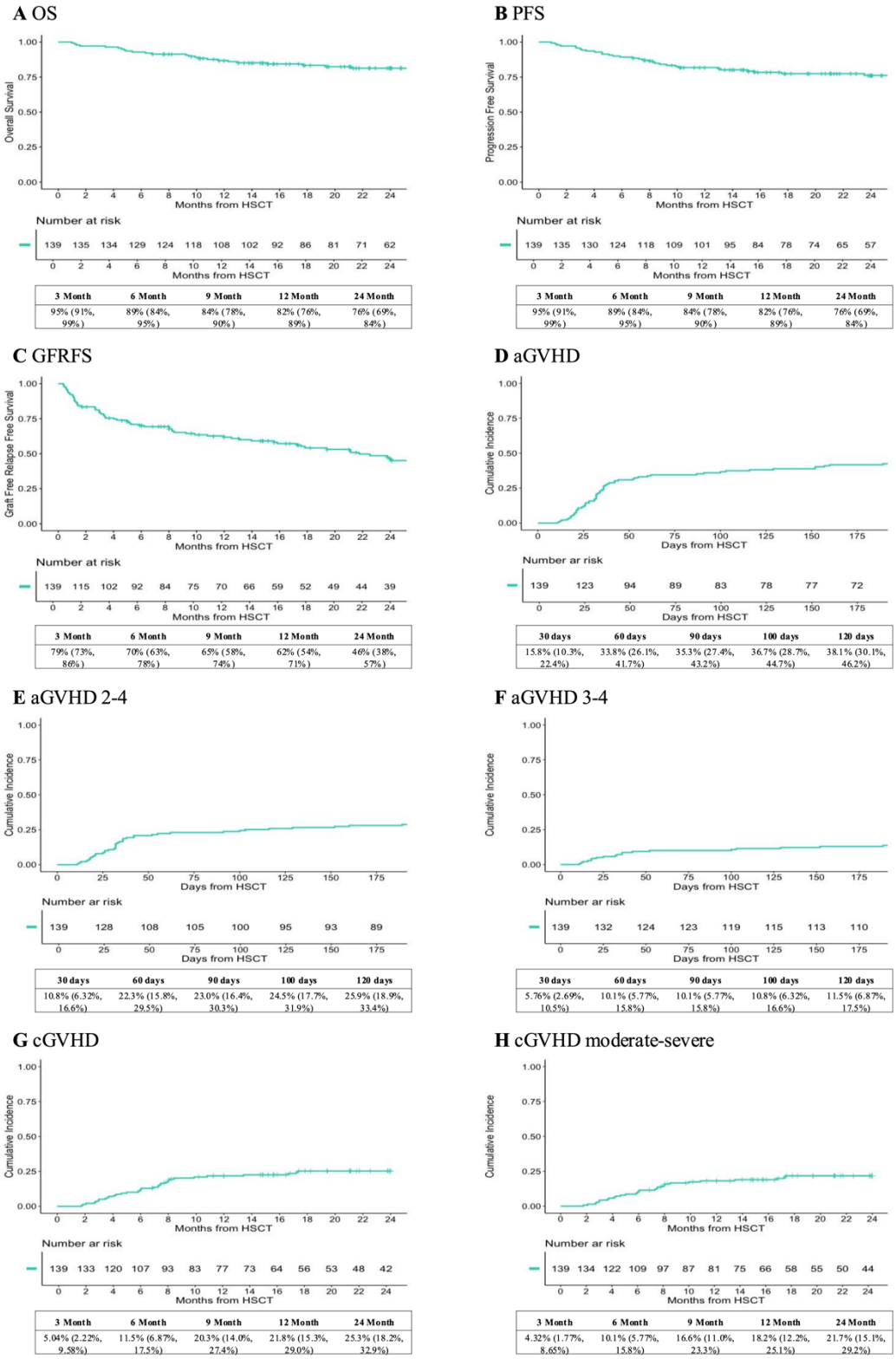
Characteristic	Exploratory Cohort N = 39[1]	Expansion Cohort N = 100[1]	Overall Population N = 139[1]
Diagnosis			
AML	16 (41%)	51 (51%)	67 (48%)
ALL	6 (15%)	16 (16%)	22 (16%)
MPN	6 (15%)	11 (11%)	17 (12%)
Others	6 (15%)	2 (2.0%)	8 (5.8%)
MDS	3 (7.7%)	8 (8.0%)	11 (7.9%)
Lymphomas	2 (5.1%)	9 (9.0%)	11 (7.9%)
CML		3 (3.0%)	3 (2.2%)
Early vs Advanced			
Early	26 (67%)	73 (73%)	99 (71%)
Advanced	13 (33%)	27 (27%)	40 (29%)
Age (years)	55 (22-71)	55 (18-70)	55 (18-71)
>60 years	14 (36%)	35 (35%)	49 (35%)
HCT-CI			
0-2	28 (72%)	50 (50%)	78 (56%)
>2	11 (28%)	50 (50%)	61 (44%)
Sex			
Male	28 (72%)	56 (56%)	84 (60%)
Female	11 (28%)	44 (44%)	55 (40%)
Stem Cell Source			
PBSC	31 (79%)	100 (100%)	131 (94%)
BM	7 (18%)		7 (5.0%)
CB	1 (2.6%)		1 (0.7%)
Donor Type			
Unrelated Donor	29 (74%)	93 (93%)	122 (88%)
Haploidentical	5 (13%)		5 (3.6%)
Matched Related Donor	4 (10%)	7 (7.0%)	11 (7.9%)
Cord Blood	1 (2.6%)		1 (0.7%)
HLA matching			
8/8	21 (54%)	70 (70%)	91 (65%)
7/8	12 (31%)	30 (30%)	42 (30%)
Haploidentical	5 (13%)		5 (3.6%)
4/6	1 (2.6%)		1 (0.7%)
TNC (10^8/Kg)	6.86 (0.33-17.03)	8.27 (2.12-17.57)	7.9 (0.3-17.6)
CD34 (10^6/Kg)	6.23 (0.17-10.35)	7.58 (2.84-13.19)	7.20 (0.17-13.19)
CD3 (10^6/Kg)	175 (1-474)	229 (39-2,217)	214 (1-2,217)
Donor Age (years)	33 (0-58)	27 (19-66)	29 (0-66)
Donor sex			
Male	28 (72%)	80 (80%)	108 (78%)
Female	11 (28%)	20 (20%)	31 (22%)
ABO matching			

Characteristic	Exploratory Cohort N = 39[1]	Expansion Cohort N = 100[1]	Overall Population N = 139[1]
Compatible	22 (56%)	42 (42%)	64 (46%)
Major Incompatibility	8 (21%)	28 (28%)	36 (26%)
Minor Incompatibility	6 (15%)	23 (23%)	29 (21%)
Mixed Incompatibility	3 (7.7%)	7 (7.0%)	10 (7.2%)
Donor CMV serostatus			
Positive	21 (54%)	49 (49%)	70 (50%)
Negative	18 (46%)	51 (51%)	69 (50%)
Recipient CMV serostatus			
Positive	32 (82%)	82 (82%)	114 (82%)
Negative	7 (18%)	18 (18%)	25 (18%)
Conditioning Intensity			
Myeloablative	25 (64%)	51 (51%)	76 (55%)
Reduced Intensity	14 (36%)	49 (49%)	63 (45%)
GVHD Prophilaxis			
ATLG-based	34 (87%)	100 (100%)	134 (96%)
PT-Cy	5 (13%)		5 (3.6%)
Plts recovery (days)	20.0 (11.0-42.0)	13.0 (10.0-31.0)	15.0 (10.0-42.0)
PMN recovery (days)	18.0 (11.0-33.0)	15.0 (10.0-28.0)	16.0 (10.0-33.0)
aGVHD	13 (33%)	49 (49%)	62 (45%)
aGVHD 2-4	10 (26%)	32 (32%)	42 (30%)
aGVHD 3-4	5 (13%)	15 (15%)	20 (14%)
cGVHD	5 (13%)	29 (29%)	34 (24%)
cGVHD moderate-severe	4 (10%)	25 (25%)	29 (21%)

[1]n (%); Median (Minimum-Maximum); Mean (Minimum-Maximum)

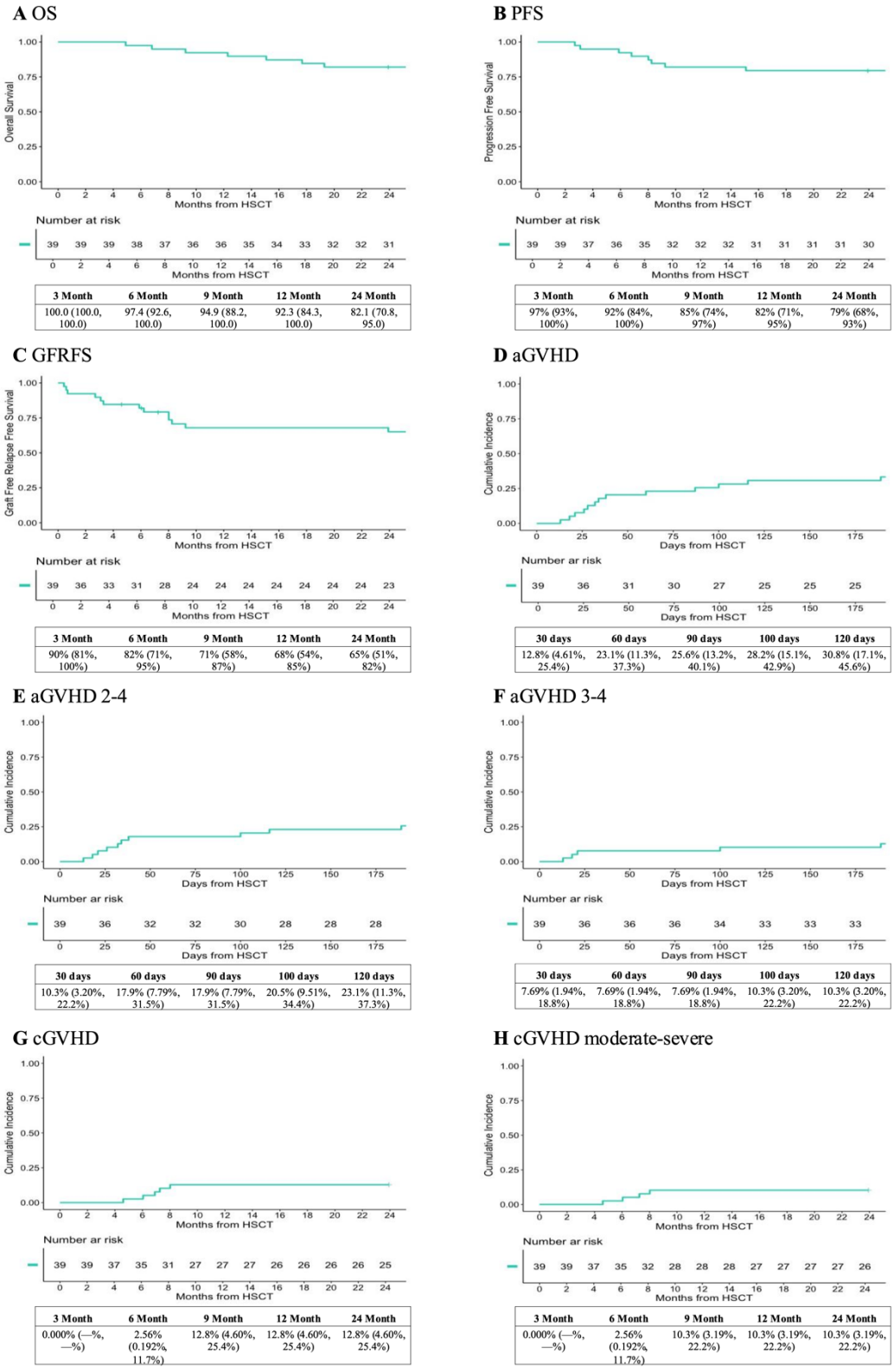
aGVHD, acute Graft Versus Host Disease; ALL, Acute Lymphoblastic Leukemia; AML, Acute Myeloid Leukemia; ATLG, Anti-T Lymphocyte Globulin; cGVHD chronic Graft Versus Host Disease; CML, Chronic Myeloid Leukemia; CMV, CitoMegaloVirus; HCT-CI, Hematopoietic

Figure 1 *Outcomes overall population*



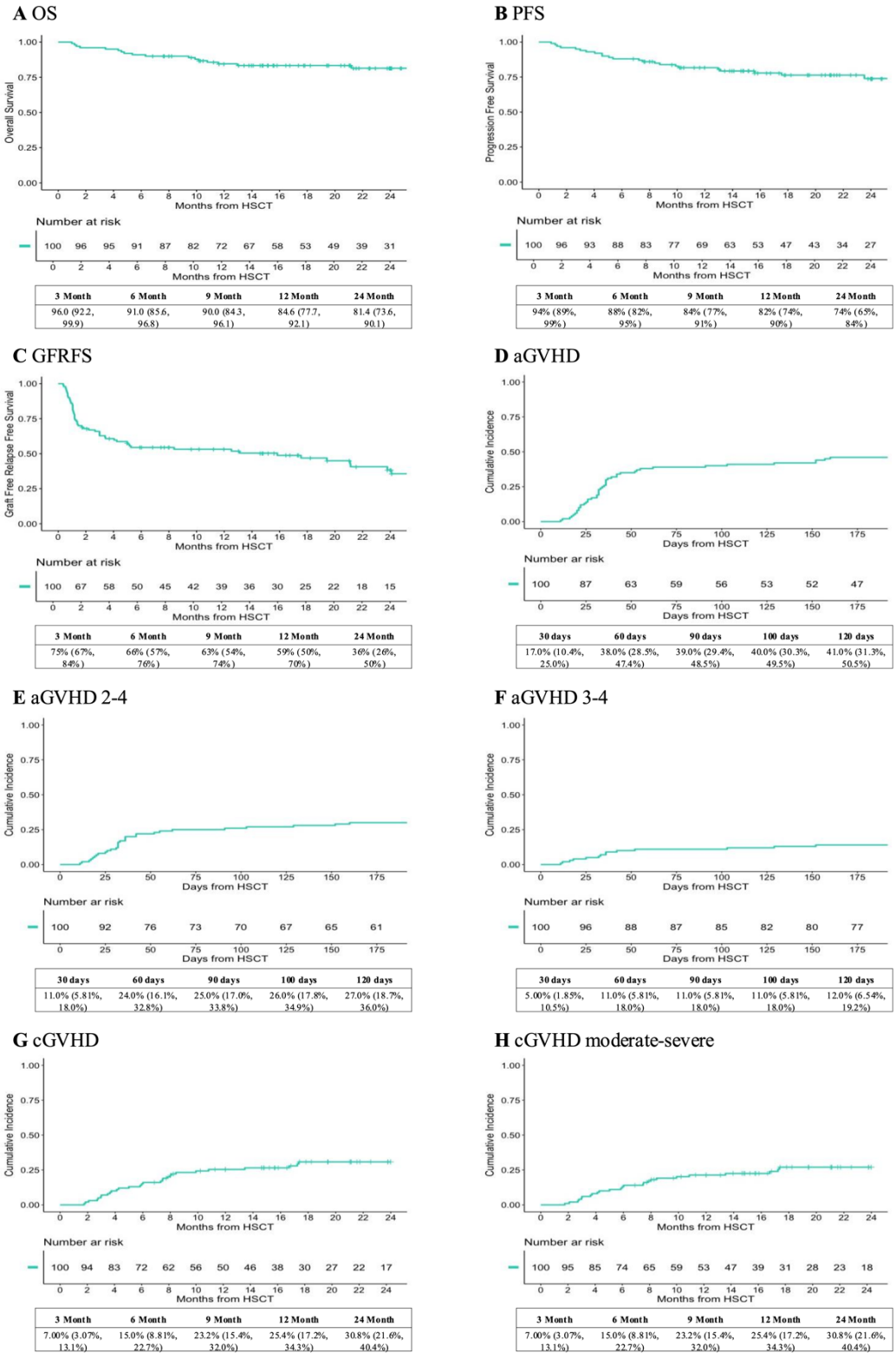
aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease; GFRFS, Graft Free Relapse Free Survival; HSCT, Hematopoietic Stem Cells Transplantation; OS, Overall Survival; PFS, Progression Free Survival

Figure 2 *Outcomes exploratory cohort*



aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease; GFRFS, Graft Free Relapse Free Survival; HSCT, Hematopoietic Stem Cells Transplantation; OS, Overall Survival; PFS, Progression Free Survival

Figure 3 *Outcomes expansion cohort*



aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease; GFRFS, Graft Free Relapse Free Survival; HSCT, Hematopoietic Stem Cells Transplantation; OS, Overall Survival; PFS, Progression Free Survival

3.2 Day-1 EVs surface antigens signature correlates with GVHD

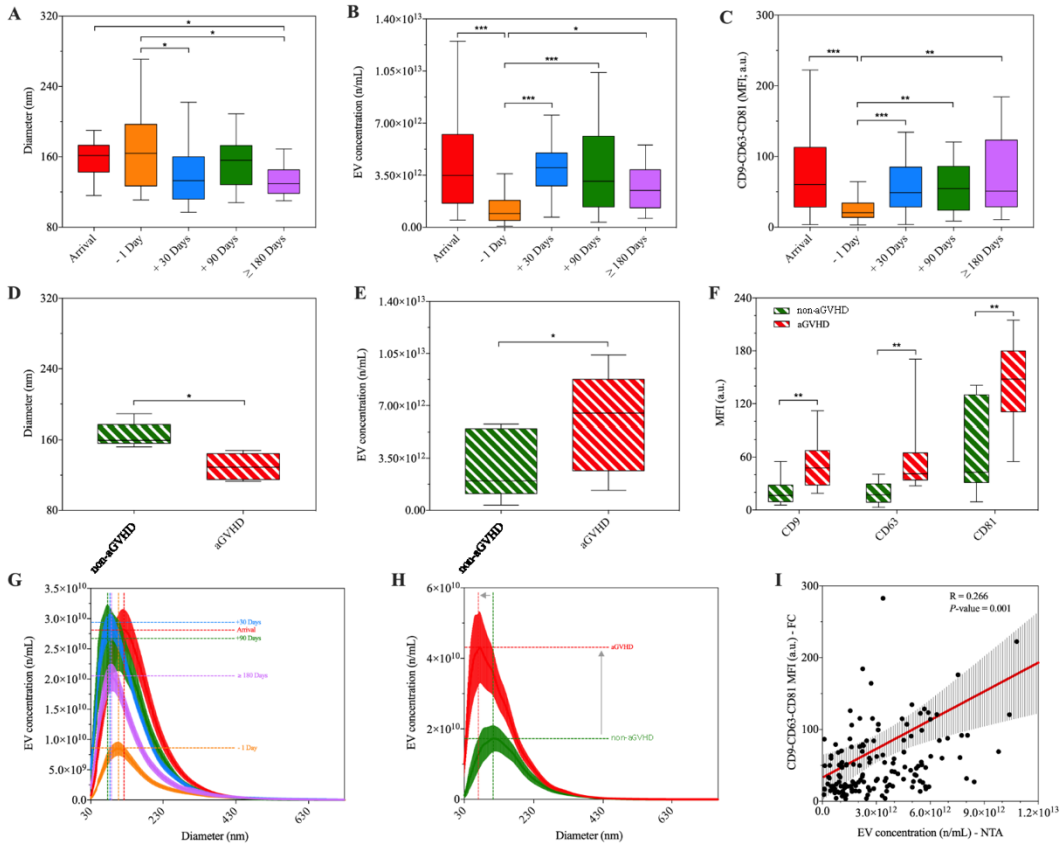
3.2.1 EVs concentration and size

The evaluation of the vesicle concentration showed a significant reduction in concentration at time point -1 compared to all other time points (***Figure 4B, G***). This difference in concentration was also confirmed by the evaluation of the mean MFI of CD9, CD63, and CD81, a measure of vesicle concentration performed via MACSPlex (***Figure 4C***).

The diameter of the vesicles was significantly different between time point -1 and +30, -1 and +180, and between pre-conditioning and +180 (***Figure 4A, G***).

In the cross-sectional comparison between patients who developed aGVHD and those who did not, at the various time points, significant differences in terms of vesicle size or concentration were found between the two groups only at time point +90. At this time point, the group of patients who developed aGVHD showed a significantly reduced vesicle diameter (median diameter [interquartile range, IQR], 129nm [115;145]) compared to the remaining patients (160 nm [156;177]) (p=0.016) (***Figure 4D, H***). Furthermore, patients with aGVHD showed an increase in vesicle concentration (median concentration [interquartile range, IQR], 6.5e12 EVs/ml [2.7e12; 8.8e12]) compared to the remaining patients (2.2e12 EVs/ml [1.1e12; 5.5e12]) (p=0.014) (***Figure 4E, H***). After stratification by vesicle size, a significant increase in the concentration of smaller vesicles (30-150 nm; p=0.013) is observed, but not for larger ones (151-500 or 501-1000 nm) in patients with aGVHD compared to the remaining patients. The increase in EVs concentration at time point +90 is also confirmed by the increase in both the MFI of CD9, CD63, and CD81 and the mean MFI among the three previous markers, measured via MACSPlex (***Figure 4F***).

Figure 4 *EVs concentration and size*



(A) *Extracellular vesicles diameter at various time points; (B) Extracellular vesicles concentration at various time points;* ***(C)*** *Mean CD9, CD63 and CD81 MFI ad various time points;* ***(D)*** *Extracellular vesicles diameter according to aGVHD (red);* ***(E)*** *Extracellular vesicles concentration according to aGVHD;* ***(F)*** *Mean CD9, CD63 and CD81 MFI according to aGVHD (red);* ***(G)*** *Vesicles concentration-diameter plot at various time points;* ***(H)*** *Vesicles concentration-diameter plot according to aGVHD (red).*

3.2.2 EVs surface epitope profile

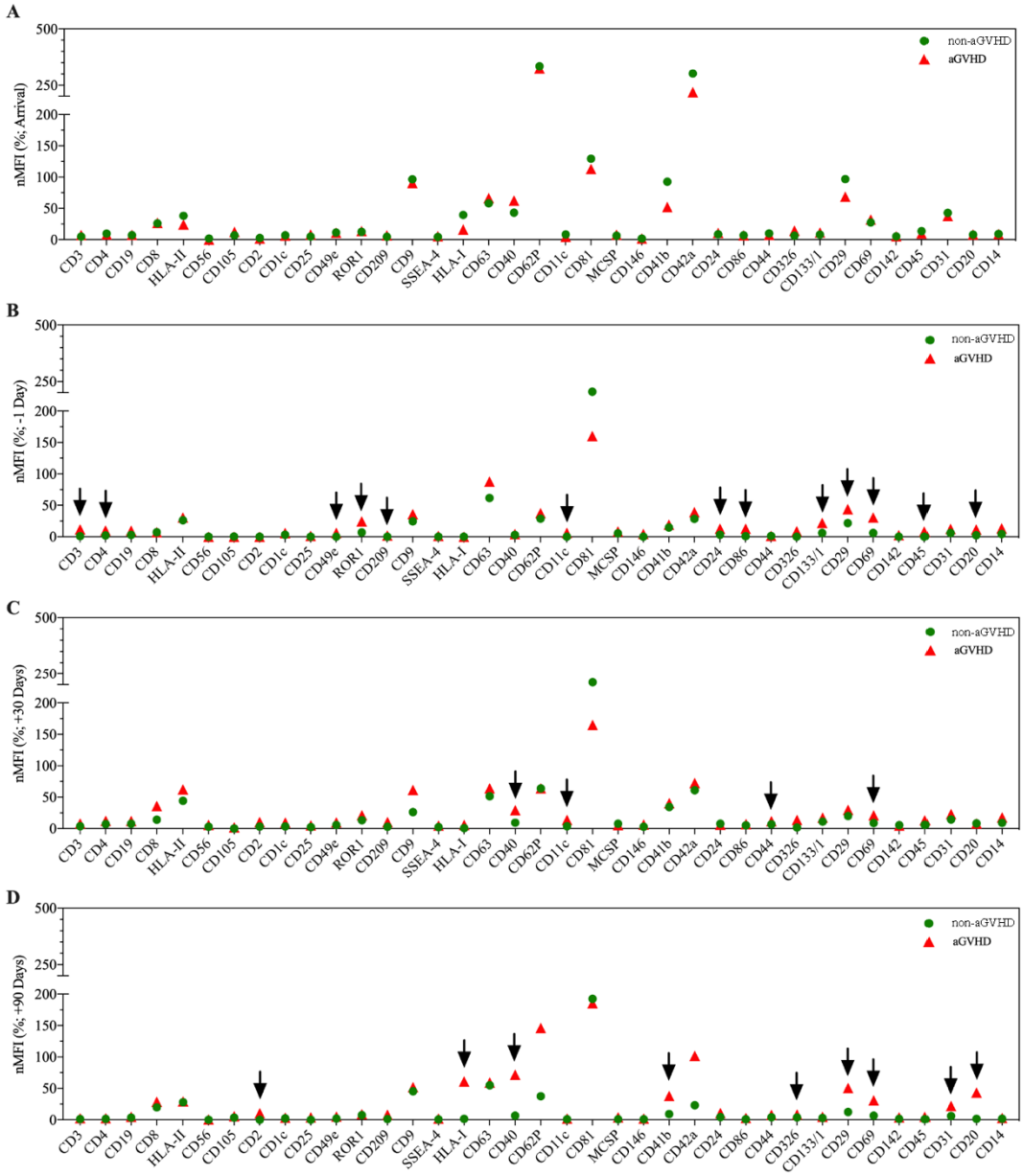
Plasma EVs surface phenotype was assessed in the exploratory cohort by MACSPlex assay, starting from the day of ward admission (arrival) an than on day -1, day + 30 and day +90 after HSC infusion. The MFI levels of 37 epitopes were analyzed for patients who developed aGVHD and for patients who did not develop aGVHD (non-aGVHD), summarized in ***Figure 5***.

At the pre-conditioning time point, no significant difference was found in the MFI of the 37 markers analyzed between the two groups (***Figure 5A***). Differently, at time point -1, a significant difference was found in the MFI levels of 13 markers, in particular CD3, CD4, CD105, CD49e, ROR1, CD209, C11c, CD24, CD86, CD133/1, CD29, CD69, CD45 and CD20 (***Figure 5B***). At time point +30, the only epitopes that maintained a different MFI in the two groups were CD11c and CD69, to which CD40 and CD44 were added (***Figure 5C***). At time point +90, a different MFI of CD40 and CD69 persists, while the epitopes CD2, CD41b, HLA-I, CD326, CD31, CD20 and CD29 are added, the latter two with a different expression already at time point -1 (***Figure 5D***). It should be noted that substantially all the significant differences between the two groups are in the sense of an increase in the expression of the markers in patients who develop aGVHD.

The CD69 is the only epitope that shows a significantly increased MFI in the aGVHD group at all time points after the start of the transplant conditioning regimen.

Each time point analyzed, except for pre-conditioning time point, is characterized by the presence of a group of markers, different for each time point, expressed differentially between patients who develop aGVHD and patients who do not develop it. Given the possible greater clinical impact of finding a potential predictive marker that can be used at an early stage in the transplant procedure, the time point -1 is of particular interest, and its differentially expressed epitopes are summarized in ***Figure 6A***. The correlation between some clinical and laboratory parameters (the latter measured at time point -1) and the vesicular expression of each of the 13 differentially expressed epitopes at time point -1 in the entire cohort was evaluated (***Figure 6B***). Overall, these data suggest a signature of circulating markers at time point -1, indicating a potential predictive tool for the onset of aGVHD.

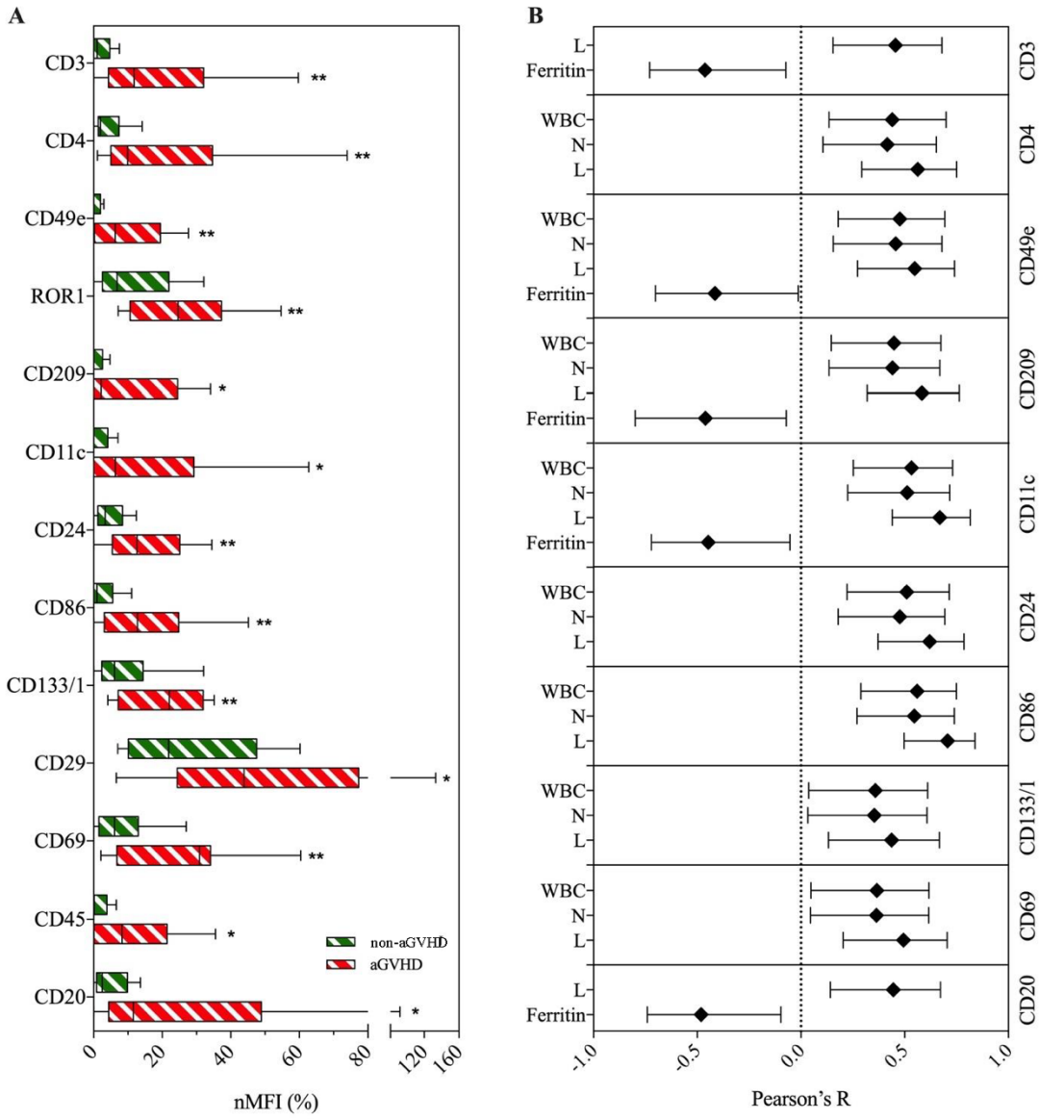
Figure 5 *EVs surface epitopes expression (aGVHD vs non-aGVHD)*



(A) *Pre-conditioning time point nMFI of 37 epitopes divided between aGVHD (red) and non-aGVHD (green);* ***(B)*** *Day -1 nMFI of 37 epitopes divided between aGVHD (red) and non-aGVHD (green);* ***(C)*** *Day +30 nMFI of 37 epitopes divided between aGVHD (red) and non-aGVHD (green);* ***(D)*** *Day + 90 nMFI of 37 epitopes divided between aGVHD (red) and non-aGVHD (green).* ***Arrows*** *indicate epitopes expressed significantly differently between patients who develop aGVHD and those who do not*

aGVHD, acute Graft Versus Host Disease; HLA, Human Leukocyte Antigen; nMFI, normalized Mean Fluorescence Intensity; MCSP, Melanoma Chondroitin Sulfate Proteoglycan; ROR1, Receptor tyrosine kinase-like Orphan Receptor 1; SSEA-4, Stage Specific Embryonic Antigen 4

Figure 6 *Cross-sectional evaluation at day -1 (aGVHD vs. non-aGVHD) and correlation with clinical parameters*



(A) *Cross-sectional evaluation at day -1 comparing aGVHD (red) with non-aGVHD (green);* ***(B)*** *correlation between clinical parameters and vesicular expression of each of the 13 differentially expressed epitopes at time point -1.*

L, Lymphocytes count; N, Neutrophils count; ROR1; WBC, With Blood Cells.

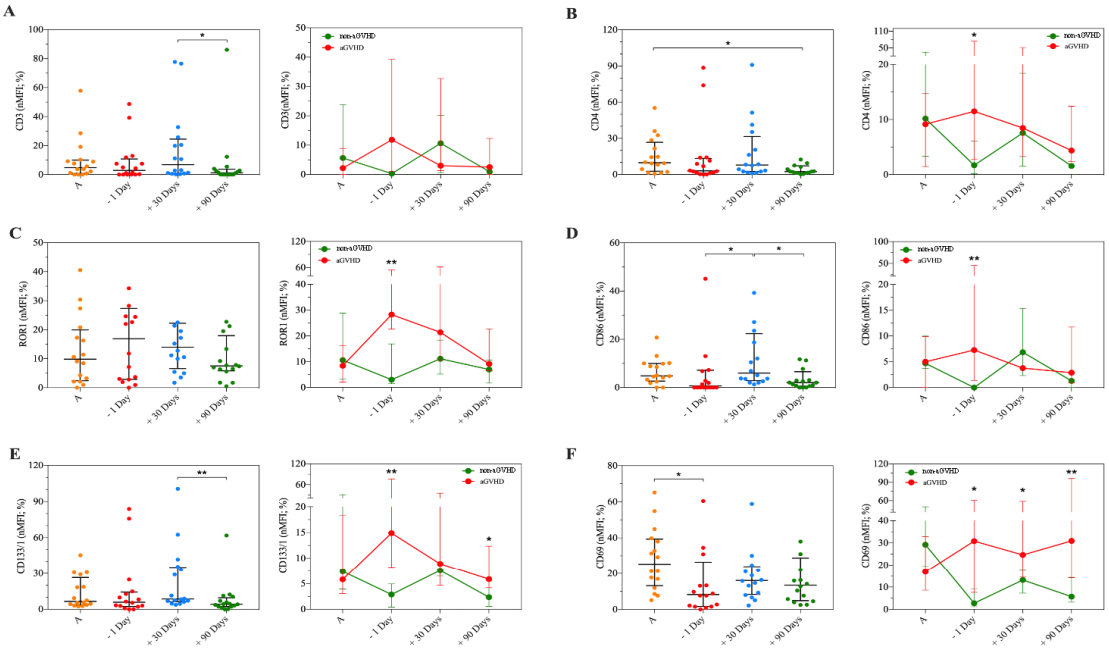
3.2.3 Longitudinal analysis

From the initial sample of 39 patients, 16 had a sample available for each time point. On these, a longitudinal analysis of surface vesicular markers was performed, stratified by the onset or not of aGVHD (7 aGVHD vs 9 noGVHD).

As in the entire cohort of 39 patients, in this selected cohort, an increase in MFI for various antigens is observed in the aGVHD group at the various time points.

At time point -1, a significant reduction in the expression of some markers is observed in patients who will develop aGVHD. In ***Figure 7***, the longitudinal trend of the 6 markers associated with aGVHD at time point -1 is highlighted. It should also be noted that CD69 was confirmed as the only marker that is significantly increased in patients who develop aGVHD at all analyzed time points except for pre-conditioning.

Figure 7 *Longitudinal analysis of the expression of selected vesicular markers (aGVHD vs non-GVHD)*



(A) *CD3 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence;* ***(B)*** *CD4 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence,* ***(C)*** *ROR1 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence;* ***(D)*** *CD86 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence;* ***(E)*** *CD133 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence;* ***(F)*** *CD69 level of expression on EVs in the longitudinal cohort at each time point and according to aGVHD occurrence.*

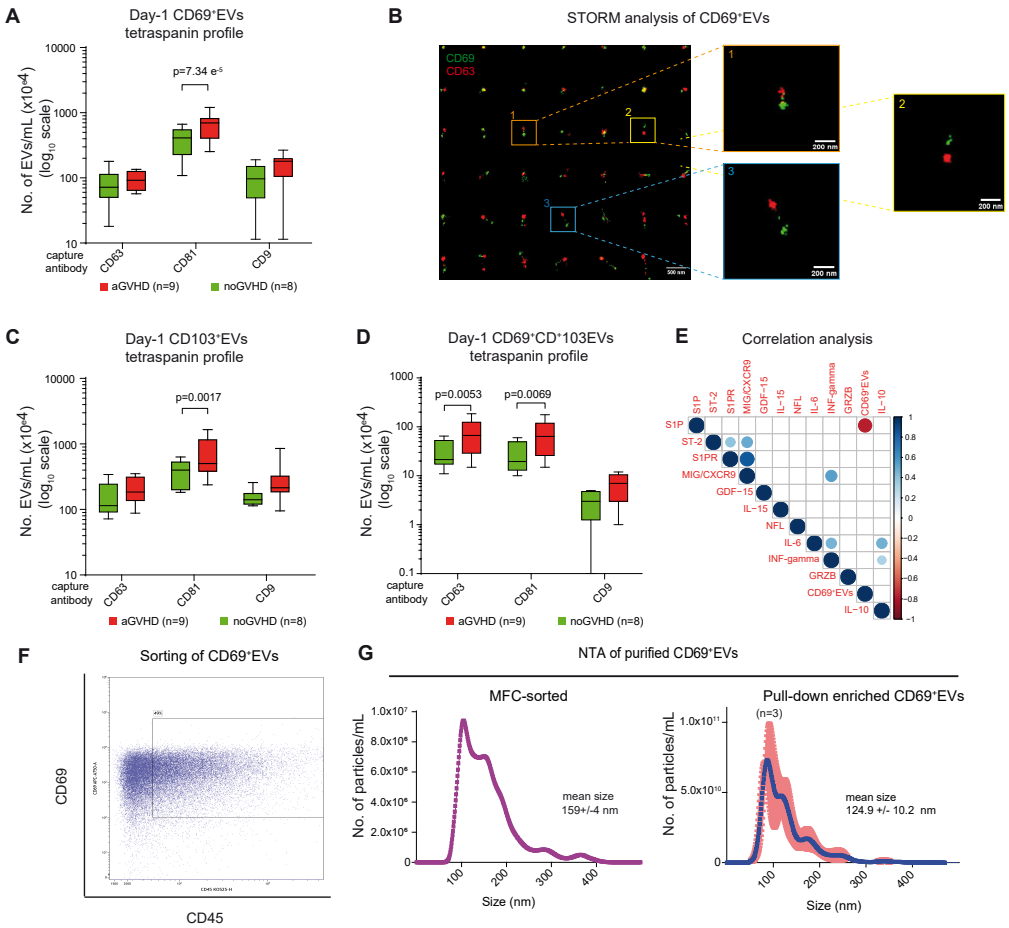
3.3 CD69+EVs characterization

3.3.1 CD69+EVs characterization as CD45+ TRM-T derived exosomes

The data above recall and support our previous data about the role of CD69+EVs in predicting GVHD occurrence [30]. CD69+EVs being elevated in GVHD patients before HSCT infusion are forcefully of host origin. The source of these vesicles is hard to attribute but we tried evaluating the hypothesis that their origin comes, at least in part, from host long-living cells (i.e., Tissue resident memory T cells, TRM-T) that have been reported to survive in the tissues after the chemotherapeutic conditioning regimen [34,35]. To prove the exosomal origin of CD69+EVs 17 patient's serum were analyzed, 8 without acute GVHD (noGVHD) and 9 with aGVHD, by ExoView platform analysis, a system that captures EVs which carry exosomal markers, namely CD63, CD9, CD81. CD69+EVs were represented along the three tetraspanins compartments (mostly in the CD81+ one) and were present at higher concentration at day-1 in aGVHD serum samples compared to noGVHD ones (**Figure 8A**). Serum CD69+EVs were also observed by STORM analysis (**Figure 8B**), which revealed the expected clustered appearance of CD69 antigen [48]. Since EVs can recapitulate the phenotype of the cells from which they originate, we assessed the presence of CD103 protein, a hallmark of TRM-T cells on CD69+ EVs [49,50]. CD103+EVs and double positive CD69+CD103+ EVs were higher in GVHD patients' serum (**Figure 8C-D**). Literature data show that CD69 expression halts the egress of TRM-T cells by impairing the Sphingosine 1-Phosphate and its cognate receptor (S1P and S1PR) axis function [50,51]. We thus measured the serum level of S1P and S1PR by ELISA test, and we found a strong negative correlation between S1PR and CD69+EVs levels (r=-0.76, p=0.00058; **Figure 8E**), but not with other systemic inflammatory markers (e.g. IL-6, IL-10, MIG/CXCL9, ST-2, IL-15, GDF-15 and NFL) [14,52–55] nor with molecules involved in the induction/maintenance of TRM-T cells (GRZB, IL-15, INF-gamma) [51,56,57] (**Figure 8E**) (28, 34, 35). Serum CD45+CD69+EVs were FACS-sorted (**Figure 8F**) and phenotypically characterized by MFC. The mean size of CD69+EVs assessed by NTA was 159+/-4 nm, typical of the exosomal compartment (**Figure 8G**), a finding confirmed by NTA analysis of EVs enriched by CD69 antigen-specific pull-down (**Figure 8G**). Overall, the data presented support our previous observation [30] that CD69

antigen identifies exosomes that carry features of the TRM-T cell compartment that are increased in the HSCT pre-transplant phase.

Figure 8 *CD69+EVs characterization as exosomes*

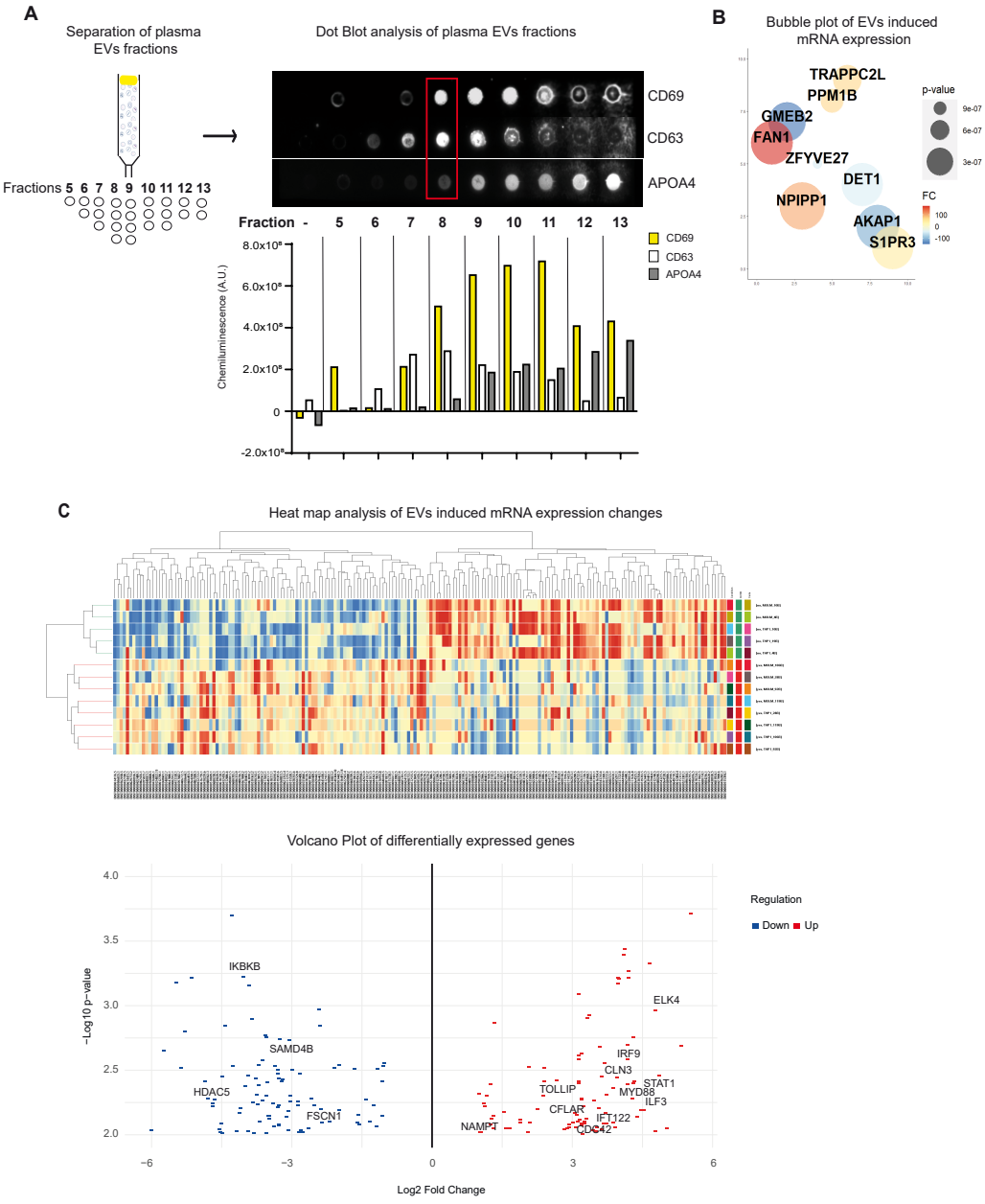


(A) *ExoView platform analysis of CD69 expression on serum EVs at day-1 in patients with aGVHD (n=9) and noGVHD (n=8) (MW test);* ***(B)*** *STORM analysis co-expression of CD63 and CD69 on patients serum EVs;* ***(C-D)*** *ExoView platform analysis of CD103 expression and CD69/CD103 co-expression on serum EVs at day-1 in patients with aGVHD (n=9) and noGVHD (n=8) (MW test);* ***(E)*** *correlation analysis between serum level of SIP, ST-2, SIPR, MIG/CXCR9, GDF-15, IL-15, NFL, IL-6, INF-gamma, GRZB, IL-10 and serum CD69+ EVs (Pearson correlation);* ***(F)*** *MFC analysis of CD69 and CD45 expression on CD69+ sorted EVs;* ***(G)*** *NTA of sorted and pulled-down CD69+EVs;*

3.3.2 CD69^{+}EVs induce a pro-inflammatory phenotype in myeloid cells.

To gain an insight into the biological activity of CD69+EVs, day-1 SEC-purified EVs Fraction-8, which contains the most of CD69+EVs (**Figure 9A**), were administered to THP-1 and MOLM-13 cells (1000 EVs/cell). After 24h, RNA-seq analysis was performed on a total of 15 samples: GVHD-derived serum EVs administered (N=4), noGVHD derived serum EVs administered (N=3) and untreated (N=1) THP1 cells; serum EVs administered (N=4), noGVHD derived serum EVs administered (N=2) and untreated (N=1) MOLM-13 cells. By comparing THP1/MOLM-13 EVs-exposed cells vs untreated cells, we found 9 differentially expressed genes (5 up- and 4 down-regulated, **Figure 9B**), sphingosine-1-phosphate receptor 3 (S1PR3) was found among the up-regulated ones, thus supporting the notion that CD69+EVs may impact the tissue egress machinery and inflammation [58]. The Reactome analysis of the 9 genes, identified 8 pathways, including "Cytokine Signaling in Immune System" (p=0.041) and "Interleukin-4 and Interleukin-13 signaling" (p=0.0097) and ALK pathway that plays a role in STING-mediated interferon signaling [59]. The RNAseq analysis of THP-1 and MOLM-13 cells exposed to GVHD derived-EVs vs noGVHD derived-EVs identified 192 differentially expressed genes (**Figure 9C**), of which 98 (51%) up- and 94 (49%) down-regulated in aGVHD EVs exposed cells. Pathway enrichment analysis on the 192 known entities showed 34 pathways (p-value ≤0.05) including "Interferon alpha/beta signaling", "Interleukin-12 signaling" and "Interleukin-6 signaling".

Figure 9 *Gene expression analysis of aGVHD or noGVHD derived EV treated cells*



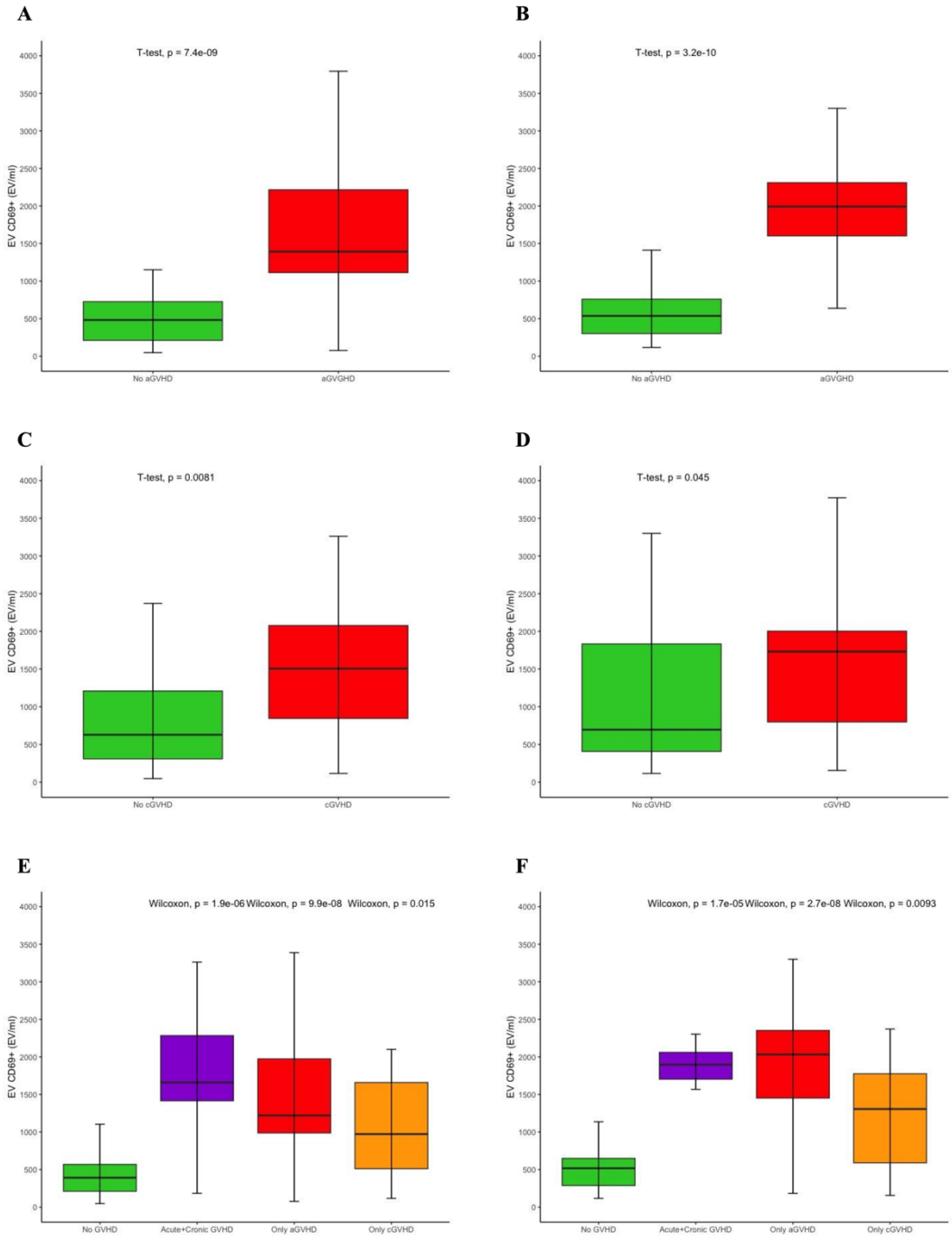
(A) *Schematic representation of experimental design of* in vitro *administration of EVs on THP-1 and MOLM-13 cells;* ***(B)*** *Bubble chart of 9 differentially expressed genes in THP-1 and MOLM-13 cells administered with EVs irrespective from their origin vs no administered ones; Bubble size and color are depictive of p-value significance and fold change;* ***(C)*** *Heat Map of 192 genes differentially expressed in THP-1 and MOLM-13 cells administered with acute GVHD and noGVHD EVs derived patients.*

3.4 CD69+EVs as predictors of acute and chronic GVHD

Prompted by the data above on the potential role of CD69+EVs as biomarker of aGVHD and cGVHD, we screened serum samples of our expansion cohort of 100 HSCT patients, collected at day-6 and day-1 before transplant by a MFC CD69+EVs detection method [30]. Dividing patients in those who developed aGVHD compared to patients who did not developed aGVHD (noGVHD), the median levels of CD69+EVs at day-6 were: 1423 (r: 77-5904) vs 483 (r: 48-2128, p=7.4e-9 **Figure 11A**), while the median levels of CD69+EVs at day-1, were: 2032 (r: 164-6025) vs 537 (r: 116-3483, p=3.2e-10). **Figure 11B**). Similarly, the median level of CD69+EVs at day -6 in patients who developed cGVHD compared to patients who did not developed cGVHD (noGVHD) were: 1531 (r: 116-4408) vs 644 (r: 48-5904, p=8.1e-3; **Figure 11C**), while the median level of CD69+EVs at day -1 were: 1761 (r: 156-4757) vs 697 (r: 116-6025, p=4.5e-2 **Figure 11D**).

Classifying the same cases in those who did not develop any form of GVHD (noGVHD) vs those who developed only aGVHD, or aGVHD and cGVHD and only cGVHD, the median level of CD69+EVs at day -6 was: noGVHD 392 (r: 48-2128) vs only aGVHD 1244 (r: 77-5904, p=6.04e-7), vs aGVHD and cGVHD 1732 (r: 184-4408, p=2.08e-7) and only cGVHD 972 (r: 116-2100, p=3.83e-2; **Figure 11E**). At day-1, the median level of CD69+EVs was: noGVHD 518 (r: 116-1721) vs only aGVHD 2037 (r: 183-6025, p=5.21e-8), vs aGVHD and cGVHD 2003 (r: 164-4757, p=6.69e-7) and only cGVHD 1306 (r: 156-3843, p=4.14e-2; **Figure 11F**).

Figure 10 *CD69+EVs levels Box Plots*

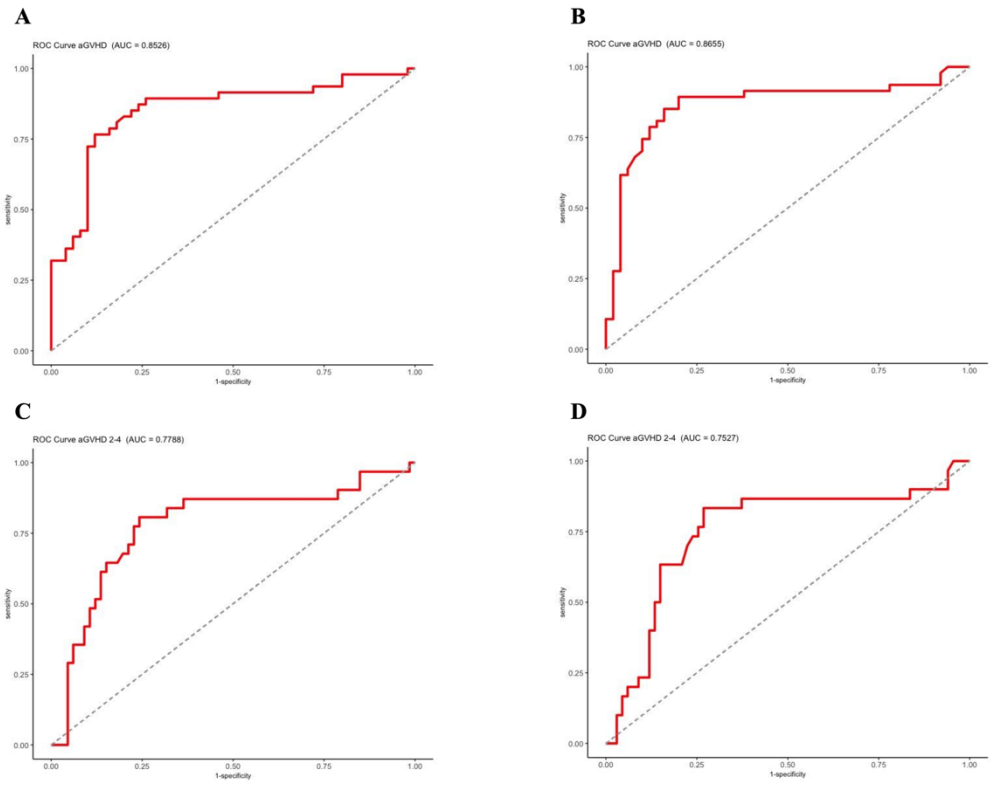


(A) *Day -6 CD69+EVs levels according to aGVHD occurrence;* ***(B)*** *Day -1 CD69+EVs levels according to aGVHD occurrence;* ***(C)*** *Day -6 CD69+EVs levels according to cGVHD occurrence;* ***(D)*** *Day -1 CD69+EVs levels according to cGVHD occurrence;* ***(E)*** *Day -6 CD69+EVs levels according to only aGVHD, only cGVHD or aGVHD and cGVHD occurrence;* ***(F)*** *Day -1 CD69+EVs levels according to only aGVHD, only cGVHD or aGVHD and cGVHD occurrence.*

aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease

ROC analyses showed that, above 1113 CD69+EVs at day-6 the onset of aGVHD is predicted with a sensitivity of 76.60% and specificity of 88.00% (**Figure 12A**), as well as that, above 1081 CD69+EVs at day-1, the onset of aGVHD is predicted with sensitivity of 89.36% and specificity of 80.00% (**Figure 12B**). AUC were 0.853 (95% CI, 0.771- 0.934) and 0.866 (95% CI, 0.783-0.948), respectively. Likewise, ROC analysis showed that the onset of cGVHD is predicted at day-6 by a level above 1393 CD69+EVs with a sensitivity of 62.07% and specificity of 82.35% (**Figure 12C**), whereas at day-1 by a level above 1200 CD69+EVs with a sensitivity of 75.86% and specificity of 60.29% (**Figure 12D**). AUC were 0.700 (95% CI, 0.581 - 0.819) and 0.669 (95% CI, 0.548-0.790) respectively.

Figure 11 *CD69+EVs ROC Curve according to aGVHD and cGVHD*



(A) *Day -6 CD69+EVs ROC curve according to aGVHD occurrence;* ***(B)*** *Day -1 CD69+EVs ROC curve according to aGVHD occurrence;* ***(C)*** *Day -6 CD69+EVs ROC curve according to cGVHD occurrence;* ***(D)*** *Day -1 CD69+EVs ROC curve according to cGVHD occurrence.*

aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease

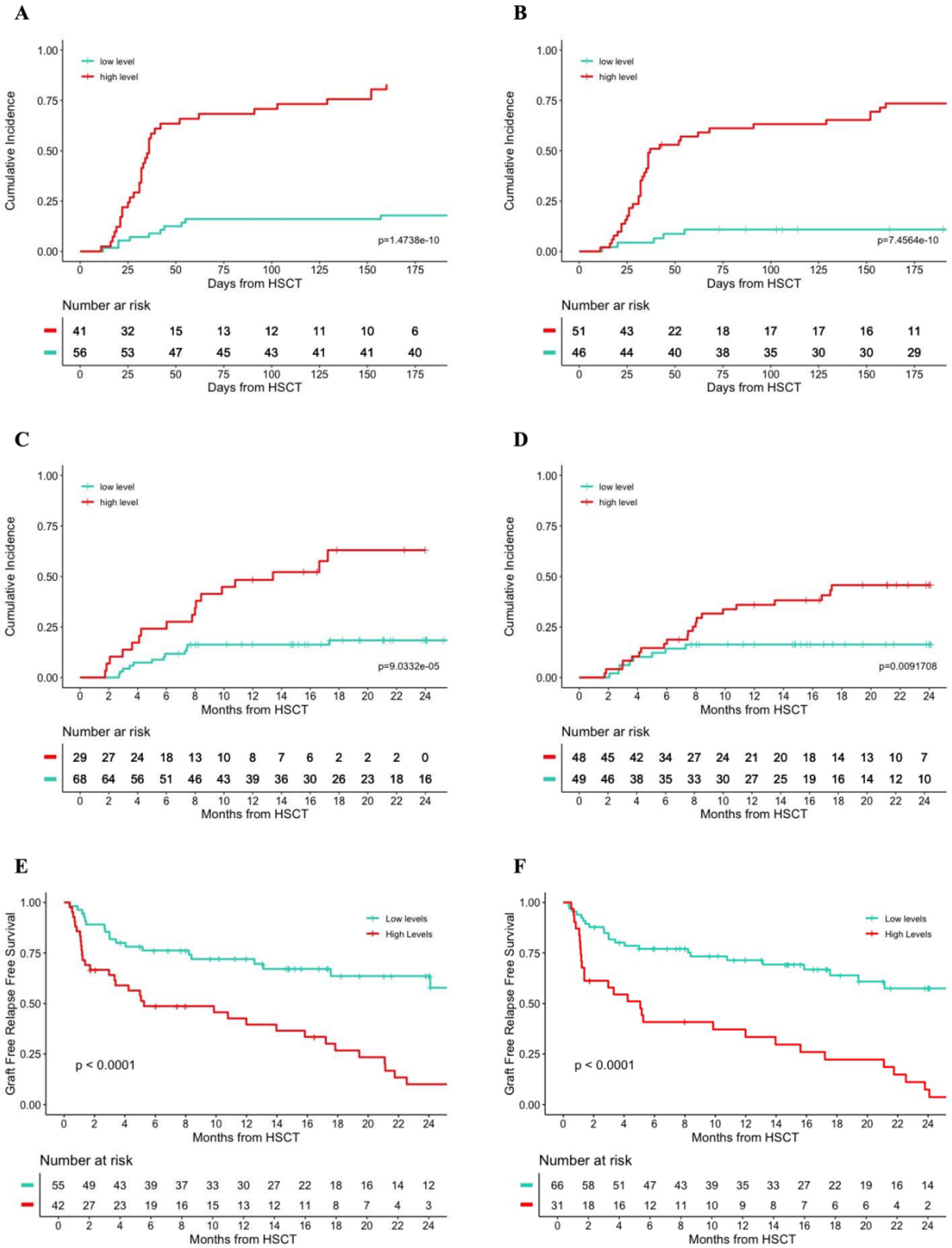
Moreover, when patients were classified as in high and low risk according to the optimal cutoff determined by ROC analysis, the amount of CD69+EVs at day-1 and day-6 resulted predictive of aGVHD CI, (HR: 6.52, [95% CI, 1.92-22.10], p=0.003; HR: 3.16, [95% CI, 1.23-8.10], p=0.017) along with HTC-CI (HR: 1.36, [95% CI, 1.16-1.60], p<0.001) and recipient sex (male sex HR: 0.40, [95% CI, 0.23-0.68], p<0.001) in multivariable analysis (**Table 2**) (Univariate analysis of clinical parameters along with CD69+EV levels is reported in **Table 3**). Accordingly aGVHD CI at day+100 in patients with higher levels of CD69+EVs at day-6 was 71% (95% CI, 54-82) compared to 16% (95% CI, 8-27) in those with lower levels of CD69+EVs (p=1.5e-10) (**Figure 13A**), in the same way patients with higher levels of CD69+EVs at day-1 showed an aGVHD CI at day+100 of 63% (95% CI, 48-75) compared to 11% (CI 95%: 4-22) in those with a lower levels of CD69+EVs (p=7.5e-10) (**Figure 13B**). Dissimilarly, only the amount of CD69+EVs at day-6 was predictive of cGVHD CI in multivariable analysis (HR: 4.17 [95% CI, 1.95-8.92], p<0.001) along with recipient age (HR: 1.03 [95% CI, 1.00-1.06], p=0.028) (**Table 2**). cGVHD CI at 1 year in patients with a level of CD69+EVs at day-6 above and below the cut-off determined by ROC analysis was 48% (95% CI, 29-65) and 16% (95% CI: 9-26) respectively (p=9.0e-5) (**Figure 13C**). Finally, we analyzed GFRFS defining a cut-off for CD69+EVs at day-6 and day-1 according to maximally selected rank statistics (day-1 CD69+EVs cut-off: 1819 EVs/µl; day-6 CD69+EVs cut-off: 1103 EVs/µl). Two years GFRFS significantly differs between patients with a lower or higher day-1 level of CD69+EVs according to cut-off; 58% (95% CI, 45-74) and 7% (95% CI, 2-28) respectively (p<0.0001). In the same way patients with day-6 level of CD69+EVs lower than defined cut-off showed a GFRFS of 64% (95% CI, 51-79) compared to 10% (95% CI, 4-29) of those with a higher level (p<0.0001) (**Figure 13E-F**). According to these results in multivariable analysis higher CD69+EVs levels at day-1 and day-6 were associated with GFRFS events (day-1 CD69+EVs HR: 3.33 [95% CI, CI 1.74-6.35], p<0.001; day-6 CD69+EVs HR: 1.99 [95% CI, 1.03-3.83], p=0.036) along with HCT-CI (HR: 1.27 [95% CI, 1.08-1.49], p=0.003) and infused CD3+ (HR: 1.00 [95% CI, 1.00-1.00], p=0.009) (**Table 2**).

Table 2 *Multivariable Analysis*

Group	Characteristic	N	HR[1]	95% CI[1]	p-value
aGVHD	HCT-CI	94	1.36	1.16, 1.60	<0.001
	Cut-off CD69+ EVs (day -1)	94			0.003
	low level		—	—	
	high level		6.52	1.92, 22.1	
	Sex	94			<0.001
	Female		—	—	
	Male		0.40	0.23, 0.68	
	Cut-off CD69+ EVs (day -6)	94			0.017
	low level		—	—	
	high level		3.16	1.23, 8.10	
cGVHD	Cut-off CD69+ EVs (day -6)	94			<0.001
	low level		—	—	
	high level		4.17	1.95, 8.92	
	Age	94	1.03	1.00, 1.06	0.028
GFRFS	HCT-CI	94	1.27	1.08, 1.49	0.003
	Cut-off CD69+ EVs (day -1)	94			<0.001
	low level		—	—	
	high level		3.33	1.74, 6.35	
	Cut-off CD69+ EVs (day -6)	94			0.036
	low level		—	—	
	high level		1.99	1.03, 3.83	
	CD3 (10^6/Kg)	94	1.00	1.00, 1.00	0.009
aGVHD 2-4	Cut-off CD69+ EVs (day -1)	94			0.026
	low level		—	—	
	high level		4.62	1.20, 17.8	
	Cut-off CD69+ EVs (day -6)	94			0.046
	low level		—	—	
	high level		3.09	1.02, 9.36	
	Sex	94			0.006
	Female		—	—	
	Male		0.32	0.14, 0.72	
	HCT-CI	94	1.31	1.06, 1.63	0.014
cGVHD moderate-severe	Cut-off CD69+ EVs (day -6)	94			<0.001
	low level		—	—	
	high level		4.52	2.03, 10.1	
	TNC (10^8/Kg)	94	1.18	1.05, 1.34	0.007

CI, Confidence Interval; CMV, CitoMegaloVirus; HLA, Human Leukocyte Antigen; HR, Hazard Ratio; HCT-CI, Hematopoietic stem Cells Transplantation Comorbidity Index; TNC, Total Nucleated Cells

***Figure 12** aGVHD, cGVHD and GFRFS according to CD69+EVs levels*

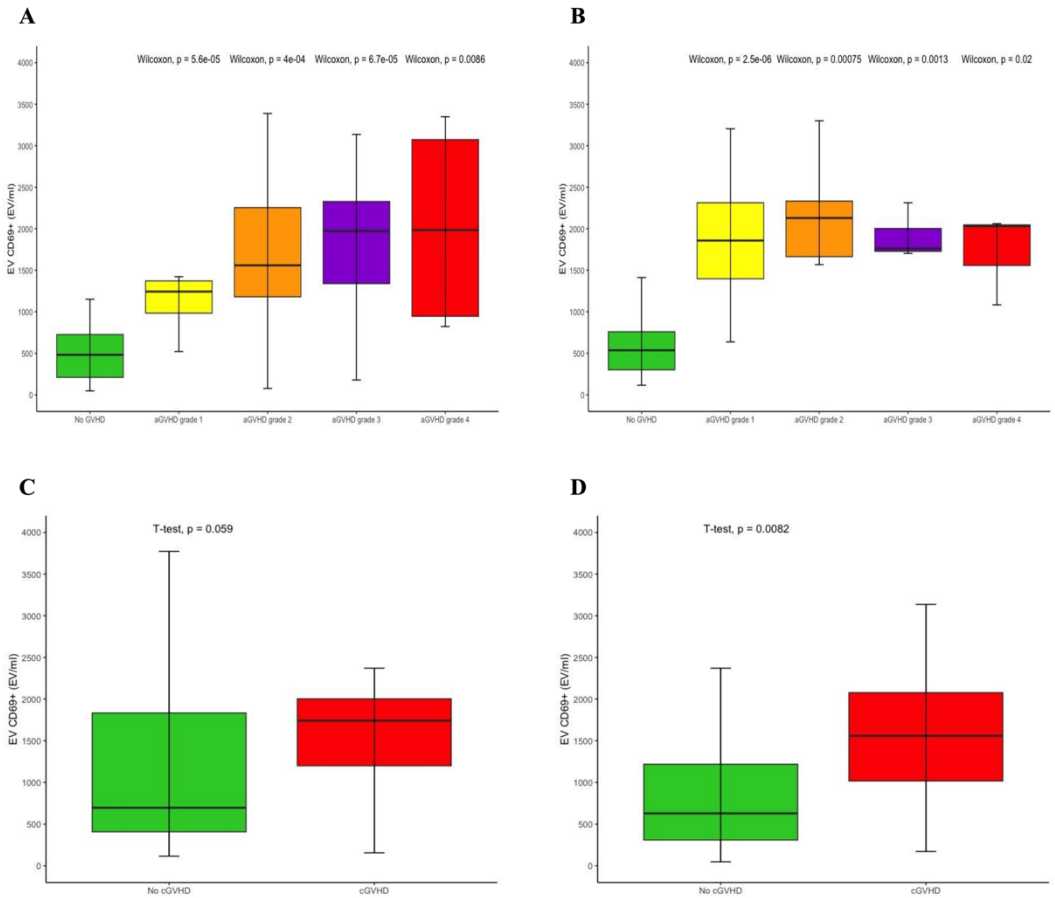


***(A)** aGVHD cumulative incidence according to higher or lower day -6 CD69+EVs according to ROC curve determined optimal cutoff; **(B)** aGVHD cumulative incidence according to higher or lower day -1 CD69+EVs according to ROC curve determined optimal cutoff; **(C)** cGVHD cumulative incidence according to higher or lower day -6 CD69+EVs according to ROC curve determined optimal cutoff;*

(D) *cGVHD cumulative incidence according to higher or lower day -1 CD69+EVs according to ROC curve determined optimal cutoff;* ***(E)*** *GFRFS according to higher or lower day -6 CD69+EVs according to ROC curve determined optimal cutoff;* ***(F)*** *GFRFS according to higher or lower day -1 CD69+EVs according to ROC curve determined optimal cutoff.*

Patients who developed aGVHD have a higher level of CD69+EVs at day -6 and -1 independently from aGVHD grade (**Figure 14A-B**). The median level of CD69+EVs at day -6 in patients who developed cGVHD moderate-severe (cGVHD) compared to patients who did not developed cGVHD moderate-severe (noGVHD) were: 1587 (r: 172-4408) vs 644 (r: 48-5904, p=0.59; **Figure 14C**), while the median level of CD69+EVs at day -1 were: 1819 (r: 156-4757) vs 697 (r: 116-6025, p=8.2e-3 **Figure 14D**).

Figure 13 *CD69+EVs levels according to aGVHD grade and cGVHD moderate-severe*

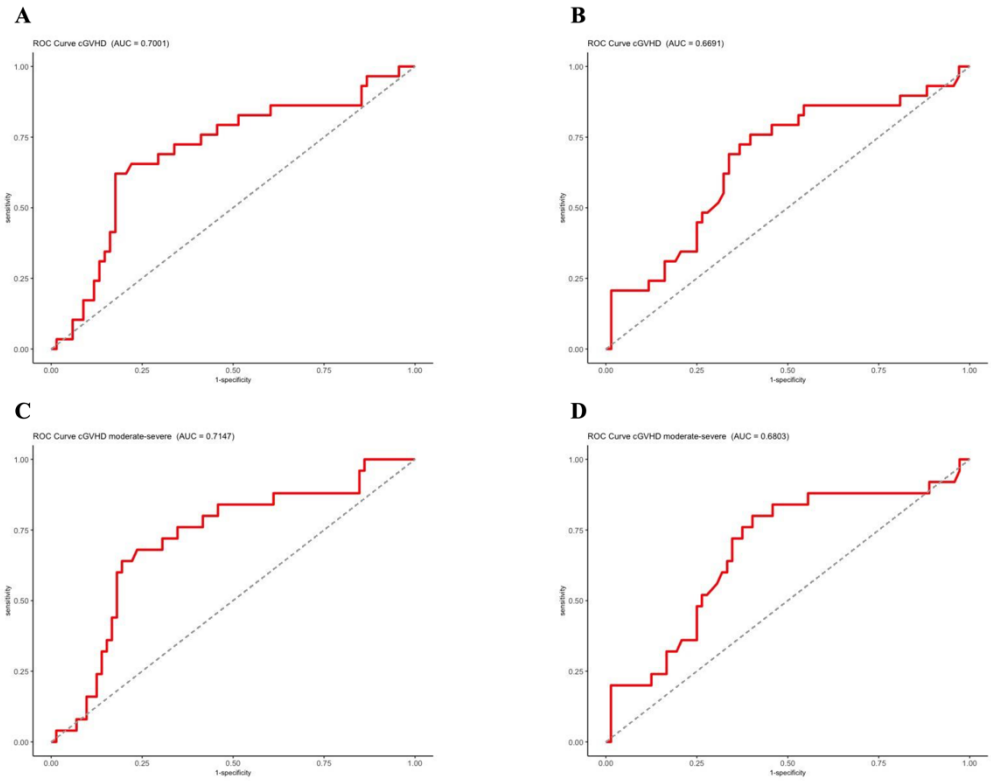


(A) *Day -6 CD69+EVs levels according to aGVHD grade;* ***(B)*** *Day -1 CD69+EVs levels according to aGVHD grades;* ***(C)*** *Day -6 CD69+EVs levels according to cGVHD moderate-severe occurrence;* ***(D)*** *Day -1 CD69+EVs levels according to cGVHD moderate-severe occurrence.*

aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease

When the occurrence of aGVHD 2-4 was considered, ROC analysis at the two time-points showed that a value above 1122 of CD69+EVs at day-6 before infusion predicted the disease with a sensitivity of 80.65% and specificity of 75.76%, whilst a value of 1568 CD69+EVs at day-1 before infusion predicted the onset of aGVHD 2-4 with a sensitivity of 83.33% and specificity of 73.13%. AUC were 0.779 (95% CI, 0.669-0.888) and 0.753 (95% CI, 0.636-0.869) respectively (**Figure 15A, B**). In the same way, cGVHD moderate-severe was predicted at day-6 by a level of CD69+EVs above 1393 with a sensitivity of 64.00% and specificity of 80.56%, while a level of 1200 CD69+EVs at day-1 was predictive of cGVHD moderate-severe with a sensitivity of 80.00% and specificity of 59.72% (**Figure 15C, D**). AUC were 0.715 (95% CI, 0.596-0.833) and 0.680 (95% CI, 0.554-0.806) respectively.

Figure 14 *CD69+EVs ROC Curve according to aGVHD II-IV and cGVHD moderate-severe*



(A) *Day -6 CD69+EVs ROC curve according to aGVHD II-IV occurrence;* ***(B)*** *Day -1 CD69+EVs ROC curve according to aGVHD II-IV occurrence;* ***(C)*** *Day -6 CD69+EVs ROC curve according to cGVHD moderate-severe occurrence;* ***(D)*** *Day -1 CD69+EVs ROC curve according to cGVHD moderate-severe occurrence.*

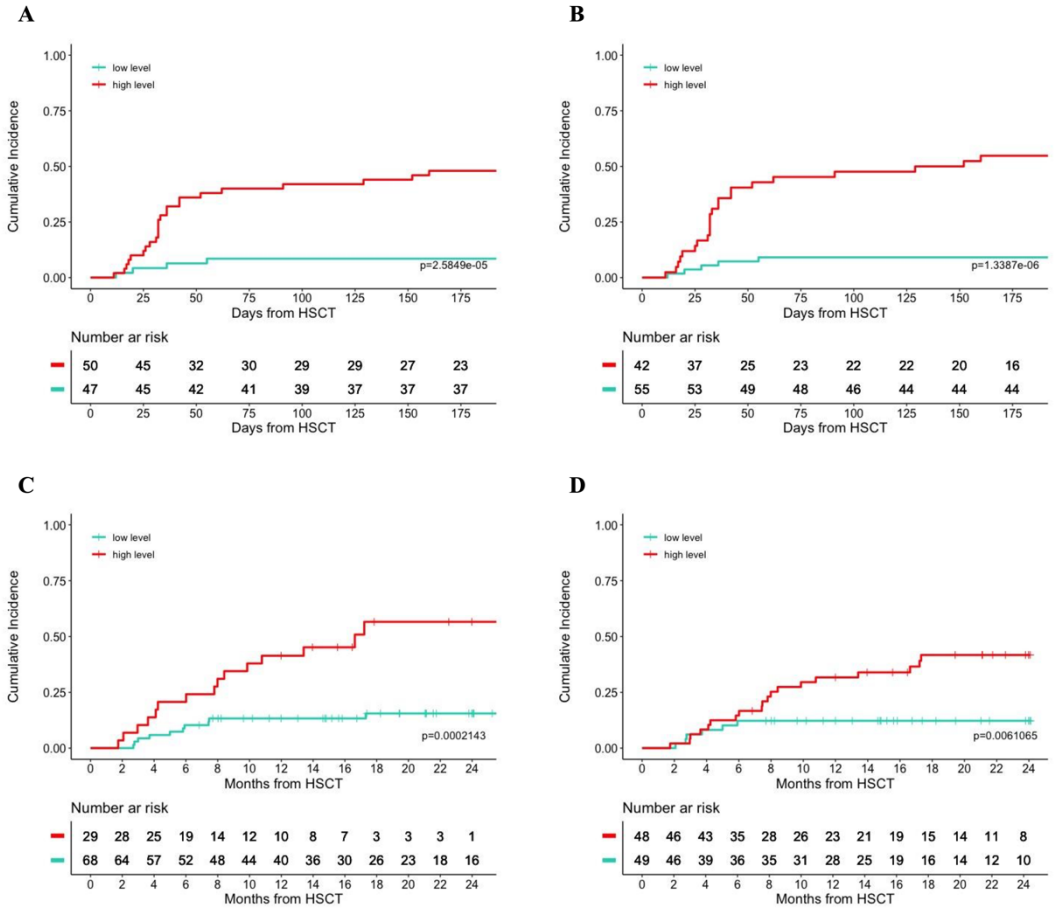
aGVHD, acute Graft Versus Host Disease; cGVHD, chronic Graft Versus Host Disease

Similarly, for aGVHD a level of CD69+EVs above the cut-off determined by ROC analysis at day-1 was correlated in multivariable analysis with aGVHD 2-4 CI (HR: 8.99, [95% CI, 3.12-25.90], p<0.001) along with HCT-CI (HR:1.30, [95% CI, 1.06-1.58], p=0.011) and recipient sex (male sex HR: 0.36, [95% CI, 0.15-0.84], p=0.018) (**Table 2**) (Univariate analysis of clinical parameters along with CD69+EV levels is reported in **Table 3**). Accordingly aGVHD II-IV CI at day+100 in patients with higher levels of CD69+EVs at day-6 was 40% (95% CI, 26-53) compared to 9% (95% CI, 3-19) in those with lower levels of CD69+EVs (p=2.6e-5) (**Figure 16A**), in the same way patients with higher levels of CD69+EVs at day-1 showed an aGVHD CI at day+100 of 45% (95% CI, 30-60) compared to 9% (CI 95%: 3-19) in those with a lower levels of CD69+EVs (p=1.3e-6) (**Figure 16B**).

On the other hand, the amount of CD69+EVs at day-6 was predictive of cGVHD moderate-severe in multivariable analysis (HR: 4.52, [95% CI, 2.03-10.10], p<0.001) along with infused total nucleated cells (HR: 1.18, [95% CI, 1.05-1.34], p=0.007) (**Table 2**). cGVHD moderate-severe CI at 1 year in patients with a level of CD69+EVs at day-6 above and below the cut-off determined by ROC analysis was 41% (95% CI, 23-59) and 13% (95% CI: 7-23) respectively (p=9.0e-5) (**Figure 16C**).

It is important to note that the different levels of CD69+EVs in the patients' groups are not paralleled by any difference in the total number of serum EVs between noGVHD vs aGVHD or cGVHD patients (**Supplemental Figure 2**).

Figure 15 *aGVHD II-IV and cGVHD according to CD69+EVs levels*



(A) *aGVHD II-IV cumulative incidence according to higher or lower day -6 CD69+EVs according to ROC curve determined optimal cutoff;* ***(B)*** *aGVHD II-IV cumulative incidence according to higher or lower day -1 CD69+EVs according to ROC curve determined optimal cutoff;* ***(C)*** *cGVHD moderate-severe cumulative incidence according to higher or lower day -6 CD69+EVs according to ROC curve determined optimal cutoff;* ***(D)*** *cGVHD moderate-severe cumulative incidence according to higher or lower day -1 CD69+EVs according to ROC curve determined optimal cutoff.*

Table 3 *Univariate Analysis*

		aGVHD			cGVHD			GFRFS			aGVHD 2-4			cGVHD moderate-severe		
Characteristic	**N**	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**
CD69+ EVs (day -6)	97	1.00	1.00, 1.00	<0.001	1.00	1.00, 1.00	0.033	1.00	1.00, 1.00	<0.001	1.00	1.00, 1.00	0.004	1.00	1.00, 1.00	0.026
Cut-off CD69+ EVs (day -6)	97			<0.001			<0.001			<0.001			<0.001			<0.001
low level		—	—		—	—		—	—		—	—		—	—	
high level		7.71	3.87, 15.4		4.13	1.99, 8.60		4.54	2.64, 7.81		7.07	2.43, 20.6		4.24	1.91, 9.37	
CD69+ EVs (day -1)	97	1.00	1.00, 1.00	<0.001	1.00	1.00, 1.00	0.010	1.00	1.00, 1.00	<0.001	1.00	1.00, 1.00	<0.001	1.00	1.00, 1.00	0.008
Cut-off CD69+ EVs (day -1)	97			<0.001			0.013			<0.001			<0.001			0.010
low level		—	—		—	—		—	—		—	—		—	—	
high level		12.5	4.82, 32.3		2.85	1.24, 6.52		4.27	2.38, 7.66		7.84	2.94, 20.9		3.39	1.33, 8.65	
Age	100	1.00	0.97, 1.02	0.7	1.04	1.01, 1.08	0.012	1.01	0.99, 1.03	0.3	1.01	0.98, 1.04	0.6	1.05	1.01, 1.09	0.012
Sex	100			0.046			>0.9			0.11			0.086			0.8
Female		—	—		—	—		—	—		—	—		—	—	
Male		0.56	0.32, 0.99		1.04	0.51, 2.13		0.66	0.39, 1.09		0.54	0.26, 1.09		0.88	0.41, 1.91	
Early vs. Advanced	100	0.75	0.39, 1.45	0.4	1.31	0.60, 2.88	0.5	0.61	0.32, 1.15	0.11	1.13	0.53, 2.40	0.7	1.69	0.75, 3.82	0.2
Conditioning Intensity	100			0.3			0.12			0.2			0.7			0.092
Myeloablative		—	—		—	—		—	—		—	—		—	—	
Reduced Intensity		0.73	0.41, 1.28		1.77	0.86, 3.65		1.42	0.84, 2.41		0.87	0.43, 1.76		1.94	0.90, 4.21	
HCT-CI	100	1.24	1.06, 1.44	0.006	1.02	0.83, 1.25	0.9	1.28	1.12, 1.47	<0.001	1.21	1.03, 1.43	0.021	0.97	0.78, 1.22	0.8
HLA matching	100			0.14			0.11			0.2			0.037			0.083
7/8		—	—		—	—		—	—		—	—		—	—	
8/8		0.64	0.35, 1.16		0.55	0.27, 1.14		0.69	0.40, 1.17		0.47	0.23, 0.96		0.50	0.23, 1.09	
TNC (10^8/Kg)	100	0.97	0.87, 1.09	0.6	1.15	1.01, 1.31	0.029	1.00	0.91, 1.09	>0.9	1.01	0.89, 1.15	0.9	1.20	1.04, 1.37	0.010
CD34 (10^6/Kg)	100	1.01	0.89, 1.14	>0.9	1.02	0.88, 1.19	0.7	1.08	0.97, 1.21	0.2	1.04	0.89, 1.23	0.6	1.07	0.91, 1.26	0.4
CD3 (10^6/Kg)	100	1.00	1.00, 1.00	0.7	1.00	1.00, 1.00	0.5	1.00	1.00, 1.00	0.14	1.00	1.00, 1.00	>0.9	1.00	1.00, 1.00	0.4
Recipient CMV serostatus	100			0.10			>0.9			0.10			0.10			0.8
Negative		—	—		—	—		—	—		—	—		—	—	
Positive		0.56	0.28, 1.12		1.05	0.40, 2.78		0.58	0.31, 1.08		0.50	0.22, 1.14		0.85	0.32, 2.29	
Donor CMV serostatus	100			0.8			0.5			0.5			0.2			0.3
Negative		—	—		—	—		—	—		—	—		—	—	
Positive		1.09	0.62, 1.92		0.79	0.38, 1.62		1.20	0.72, 1.99		0.63	0.31, 1.29		0.64	0.29, 1.40	
Donor Age	100	1.02	0.98, 1.05	0.3	0.99	0.96, 1.03	0.6	1.01	0.99, 1.04	0.4	1.01	0.97, 1.05	0.7	0.99	0.95, 1.03	0.5
Donor Sex	100			0.4			0.3			0.5			0.6			0.5
Female		—	—		—	—		—	—		—	—		—	—	
Male		0.77	0.40, 1.49		1.70	0.59, 4.93		1.22	0.64, 2.30		1.29	0.50, 3.35		1.39	0.47, 4.10	

CI, Confidence Interval; CMV, CitoMegaloVirus; HLA, Human Leukocyte Antigen; HR, Hazard Ratio; HCT-CI, Hematopoietic stem Cells Transplantation Comorbidity Index; TNC, Total Nucleated Cells

Chapter 4 - Discussion

GVHD is the most relevant complication of allogeneic stem cell transplantation. It is well known that HLA-matching, conditioning regimen, sex mismatch and GVHD prophylaxis are among the most relevant factors determining the probability of GVHD developing. However, the real occurrence of GVHD is partly unpredictable as, between patients with the same known risk factors, some develop GVHD, and others do not.

The classic pathophysiological model of aGVHD sees the donor's alloreactive T lymphocytes infused at the time of transplant as the main player in this complication. Classically, three phases are recognized that lead to the clinical manifestation of the complication [60]; Phase I consists in the activation of the host's antigen presenting cells (APCs) with an increase in the expression of surface MHC molecules and costimulatory molecules, in response to the damage caused in the peripheral tissues by conditioning regimens, immunosuppressive drugs and infectious insults [61]. Phase II is the core of classical GVHD pathogenesis and happens when APCs activate alloreactive donor T cells interacting with them in the primary lymphoid organs. The “danger” signals generated in Phase I augment this activation at least in part by increasing the expression of costimulatory molecules[62]. HLA differences between host and donor are responsible for the interaction between APC and alloreactive T cells, with CD8+ cells responding to HLA class I differences and CD4+ cells responding to HLA class II differences, but even in HLA matched transplants both CD4+ cells and CD8+ cells can be activated in response to minor histocompatibility antigens differences [63]. Donor alloreactive T cells activation is followed by a Th1 differentiation that brings an increased production of proinflammatory cytokines (IFN-γ, IL-2 and TNF-α). Phase III is the effector phase, when the tissue damage takes place. Chemokines direct the migration of donor T cells from lymphoid tissues to the target organs where they cause damage.

Peripheral tissue damage brings to amplification and propagation of the “cytokine storm” characteristic of acute GVHD; cytokines that activates APCs and enhance alloantigen presentation; recruit effector cells to target organs via the induction of inflammatory chemokines and directly cause tissue necrosis [64].

Although donor lymphocytes are the key factor of GVHD and depleting them by ex vivo manipulation prevents GVHD to take place, it is conceivable that there are host-related characteristics, which may determine the risk of this complication.

In this regard, recent literature reported that a population of memory T cells, namely tissue resident memory T cells, are involved in acute GVHD occurrence [33–35].

In this analysis, the role of extracellular vesicles was evaluated with the aim of assessing their possible use as a biomarker for GVHD. Extracellular vesicles can be produced by any cell type and have proven to be an excellent marker in many fields, both oncological and non-oncological [25]. They can also represent an epiphenomenon of biological cellular events involved in the pathogenesis of diseases, making them excellent biomarker candidates.

To evaluate the possible application of extracellular vesicles as biomarkers and to confirm our previous observation of an association between CD69+EV and the onset of GVHD [30], we conducted an analysis of the physical and surface properties of extracellular vesicles on an exploratory cohort of 39 patients who underwent serial blood draws at day -6 before HSCT (which was before conditioning), day -1 before HSCT, day +30 and day + 90 after HSCT.

Patients who developed aGVHD were compared to those who did not regarding the MFI of 37 surface markers in extracellular vesicles, analyzed using the MACSPlex method at various time points. For each time point analyzed, except for pre-conditioning time point, a significantly different MFI was found between the two groups for several markers. This observation suggests that the alteration of surface marker expression on the vesicles is secondary to the transplant procedure and not related to the intrinsic conditions of the patients in the two cohorts. The most interesting observation is the presence of a different expression of 13 markers (namely CD3, CD4, CD105, CD49e, ROR1, CD209, C11c, CD24, CD86, CD133/1, CD29, CD69, CD45 and CD20) on EVs between patients who developed aGVHD and those who did not, already at time point -1 when the donor T cells have not been infused yet.

To further investigate this observation, we conducted a longitudinal analysis on 18 patients (7 aGVHD and 9 noGVHD) for whom all samples were available at all time points. This analysis highlighted CD69 as the only markers differentially expressed in patients developing aGVHD trough all time points confirming our previous observation of an association of CD69+EV and aGVHD occurrence [30].

The possible association between CD69+EVs at day -1 and aGVHD is of interest from both a pathophysiological and a clinical point of view. From the biological point of view, knowing that extracellular vesicles potentially recapitulate the characteristics of

the cell of origin, and that this cell of origin necessarily belongs to the host (as the CD69+EVs were collected before the transplant), this generates the hypothesis that there is a cellular component belonging to the host that favors the onset of GVHD.

From a clinical point of view a marker present at such an early time point is of interest because it can potentially be used to tailor GVHD prophylaxis according to GVHD risk. Coming from the observation in the exploratory cohort we focused on pre-HSCT CD69+EVs trying to elucidate their origin and to confirm their role as a predictive biomarker in an independent cohort of 100 patients.

We moved from the previous description of a subset of T cells belonging to the host located on peripheral tissues and able to resist to lymphodepleting action of conditioning regimens. These cells called tissue resident T cells have been recently shown to contribute to GVHD pathogenesis [30,33–35].

Among the markers detected, we extended our previous report on the up-regulation of serum CD45+CD69+EVs in GVHD patients before HSCT infusion, and we also substantiate that a fraction of CD69+EVs also carry for the TRM-T cell markers CD103 [30]. In keeping with this observation, CD69+CD45+ EVs recapitulate the lineage of memory T cells. Hence, our data are in line with the tenet of the origin of CD69+CD103+CD45+ EVs from memory T cells. As far as the tissue resident memory T cell phenotype is concerned, we also provide the evidence that the serum level of day-1 sphingosine-1-phosphate, which is the major modulator of tissue resident T cell egress, is inversely correlated with serum CD69+EVs. Because this machinery is involved in T cell memory trafficking, we put forward that this finding mimics a patient-specific make-up concerning the trafficking of tissue resident T cells, that is likely to be involved in the inter-individual liability towards GHVD. Nevertheless, CD69+EVs origin warrants further investigations, since we were not able to observe TRM-T in patients' samples and although our observations suggest a possible origin of CD69+EVs from TRM-T cells more evidence are needed to definitively establish this connection. Moreover, a proportion of CD69+EVs does not show CD45 antigen and may therefore stem from other cell lineages.

To gain an insight into the biological activity of CD69+EVs, we administered day-1 SEC-purified EVs Fraction-8 THP-1 and MOLM cells. CD69+ enriched EVs triggered the mRNA expression of S1P3, a gene which is associated with a pro-inflammatory phenotype in macrophages [58]. It is also relevant to note, that the release of CD69+EVs

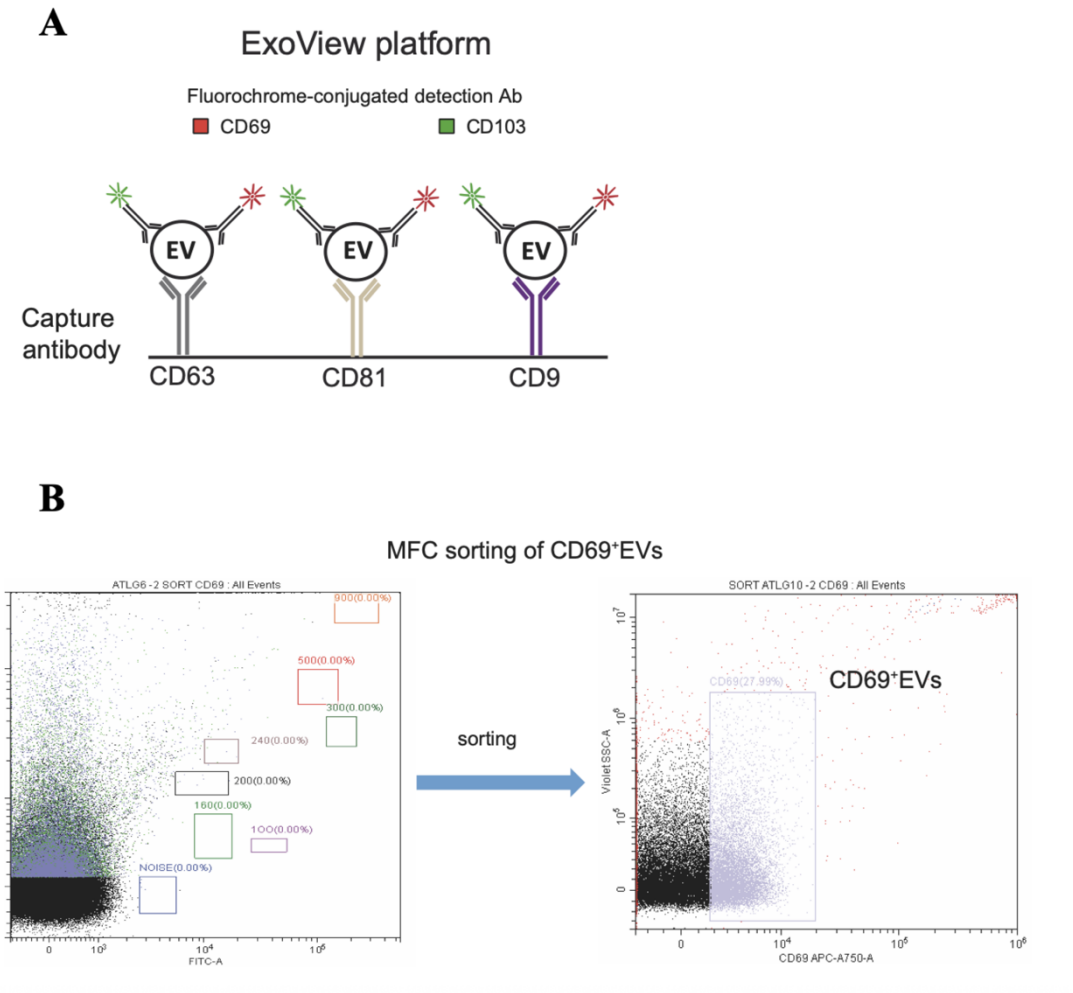
could be considered a signal of T cell activation, which can be a hint of higher levels of TRM-T cells or, even, a more activated TRM-T cells both predisposing conditions of GVHD.

Eventually we tried to test CD69+EVs measured pre transplant as an early marker of GVHD by measuring them at day -6 and day -1 in a cohort of 100 patients with a cytofluorimetric more processive method [30]. In multivariable model CD69+EV measured at both day -6 and day -1 were associated with aGVHD, aGVHD II-IV and GFRFS, while only those measured at day -6 were associated with cGVHD and cGVHD moderate-severe. According to ROC curve analysis the best diagnostic performance was that of CD69+EVs measured at day -1 in predict aGVHD with an AUC of 0.866, a sensitivity of 89.36% and a specificity of 80.00%

Hence, we here propose that the marker is suggestive for the contribution of recipient CD69 TRM-T cells in GVHD, from which CD69+EVs can be shed in serum testifying to a predisposing condition for the onset of GVHD before infusing donor cells. Such evidence expands the landscape of predictive biomarkers of GVHD. But, differently from these biomarkers, CD69+EVs appear to predict GVHD at a time prior to interaction with donor cells, thus representing a powerful tool to assess the recipient makeup and its role in the onset of HSCT complications.

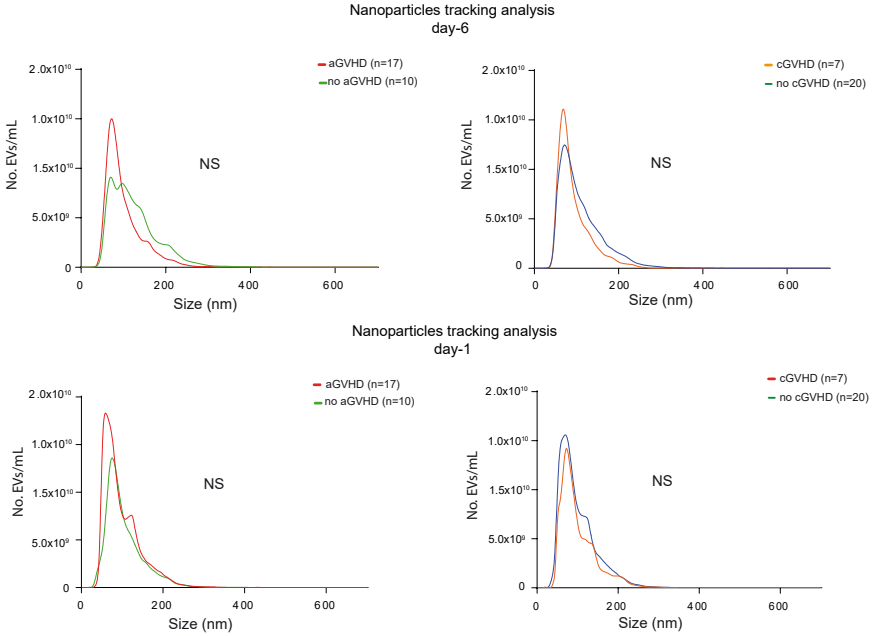
Chapter 5 - Supplemental Materials

Supplemental Figure 1 *MFC sorting of CD69^{+}EVs and schematic of ExoView platform analysis.*



(A) *Schematic representation of ExoView platform analysis of serum EVs;* ***(B)*** *Schematic representation of MFC sorting of CD69+EVs.*

Supplemental Figure 2 *Nanoparticle tracking analysis of day-6 and day-1 total serum EVs.*



Graph representing size distribution of EVs measured by NTA in serum samples of acute GVHD (n=17) and no GVHD (n=10) patients at: day-6 (A) and day-1 (B); The size range of EVs is reported in nanometers (nm).

NS, not significant.

Chapter 6 – Bibliography

[1] M. Jagasia *et al.*, 'Risk factors for acute GVHD and survival after hematopoietic cell transplantation', *Blood*, vol. 119, no. 1, pp. 296–307, Jan. 2012, doi: 10.1182/BLOOD-2011-06-364265.

[2] A. C. Harris *et al.*, 'International, multi-center standardization of acute graft-versus-host disease clinical data collection: a report from the MAGIC consortium', *Biol Blood Marrow Transplant*, vol. 22, no. 1, p. 4, Jan. 2015, doi: 10.1016/J.BBMT.2015.09.001.

[3] S. Paczesny *et al.*, 'A biomarker panel for acute graft-versus-host disease', *Blood*, vol. 113, no. 2, pp. 273–278, Jan. 2009, doi: 10.1182/BLOOD-2008-07-167098.

[4] S. Paczesny *et al.*, 'Elafin Is a Biomarker of Graft-Versus-Host Disease of the Skin', *Sci Transl Med*, vol. 2, no. 13, Jan. 2010, doi: 10.1126/SCITRANSLMED.3000406.

[5] J. E. Levine *et al.*, 'Acute graft-versus-host disease biomarkers measured during therapy can predict treatment outcomes: a Blood and Marrow Transplant Clinical Trials Network study', *Blood*, vol. 119, no. 16, pp. 3854–3860, Apr. 2012, doi: 10.1182/BLOOD-2012-01-403063.

[6] M. T. Vander Lugt *et al.*, 'ST2 as a Marker for Risk of Therapy-Resistant Graft-versus-Host Disease and Death', *New England Journal of Medicine*, vol. 369, no. 6, pp. 529–539, Aug. 2013, doi: 10.1056/NEJMOA1213299.

[7] D. M. Ponce *et al.*, 'High day 28 ST2 levels predict for acute graft-versus-host disease and transplant-related mortality after cord blood transplantation', *Blood*, vol. 125, no. 1, pp. 199–205, Jan. 2015, doi: 10.1182/BLOOD-2014-06-584789.

[8] R. P. Nelson *et al.*, 'Prognostic biomarkers for acute graft-versus-host disease risk after cyclophosphamide-fludarabine nonmyeloablative allotransplantation', *Biol Blood Marrow Transplant*, vol. 20, no. 11, pp. 1861–1864, Nov. 2014, doi: 10.1016/J.BBMT.2014.06.039.

[9] C. G. Kanakry *et al.*, 'Plasma-derived proteomic biomarkers in human leukocyte antigen-haploidentical or human leukocyte antigen-matched bone marrow transplantation using post-transplantation cyclophosphamide', *Haematologica*, vol. 102, no. 5, pp. 932–940, Apr. 2017, doi: 10.3324/HAEMATOL.2016.152322.

[10] J. E. Levine *et al.*, 'A PROGNOSTIC SCORE FOR ACUTE GRAFT-VERSUS-HOST DISEASE BASED ON BIOMARKERS: A

MULTICENTER STUDY', *Lancet Haematol*, vol. 2, no. 1, p. e21, 2015, doi: 10.1016/S2352-3026(14)00035-0.

[11] G. B. McDonald, L. Tabellini, B. E. Storer, R. L. Lawler, P. J. Martin, and J. A. Hansen, 'Plasma biomarkers of acute GVHD and nonrelapse mortality: predictive value of measurements before GVHD onset and treatment', *Blood*, vol. 126, no. 1, pp. 113–120, Jul. 2015, doi: 10.1182/BLOOD-2015-03-636753.

[12] P. Rodriguez-Otero *et al.*, 'Fecal calprotectin and alpha-1 antitrypsin predict severity and response to corticosteroids in gastrointestinal graft-versus-host disease', *Blood*, vol. 119, no. 24, pp. 5909–5917, Jun. 2012, doi: 10.1182/BLOOD-2011-12-397968.

[13] M. J. Hartwell *et al.*, 'An early-biomarker algorithm predicts lethal graft-versus-host disease and survival', *JCI Insight*, vol. 2, no. 3, p. e89798, Feb. 2017, doi: 10.1172/JCI.INSIGHT.89798.

[14] H. Major-Monfried *et al.*, 'MAGIC biomarkers predict long-term outcomes for steroid-resistant acute GVHD', *Blood*, vol. 131, no. 25, pp. 2846–2855, Jun. 2018, doi: 10.1182/BLOOD-2018-01-822957.

[15] M. H. Jagasia *et al.*, 'National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report', *Biology of Blood and Marrow Transplantation*, vol. 21, no. 3, pp. 389-401.e1, Mar. 2015, doi: 10.1016/j.bbmt.2014.12.001.

[16] S. Sarantopoulos *et al.*, 'Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease', *Blood*, vol. 113, no. 16, pp. 3865–3874, 2009, doi: 10.1182/BLOOD-2008-09-177840.

[17] H. T. Greinix *et al.*, 'Elevated Numbers of Immature/Transitional CD21- B Lymphocytes and Deficiency of Memory CD27+ B Cells Identify Patients with Active Chronic Graft-versus-Host Disease', *Biology of Blood and Marrow Transplantation*, vol. 14, no. 2, pp. 208–219, Feb. 2008, doi: 10.1016/j.bbmt.2007.10.009.

[18] Z. Kuzmina *et al.*, 'CD19(+)CD21(low) B cells and patients at risk for NIH-defined chronic graft-versus-host disease with bronchiolitis obliterans syndrome', *Blood*, vol. 121, no. 10, pp. 1886–1895, Mar. 2013, doi: 10.1182/BLOOD-2012-06-435008.

[19] H. T. Greinix *et al.*, 'CD19+CD21low B Cells and CD4+CD45RA+CD31+ T Cells Correlate with First Diagnosis of Chronic Graft-versus-Host

Disease', *Biology of Blood and Marrow Transplantation*, vol. 21, no. 2, pp. 250–258, Feb. 2015, doi: 10.1016/j.bbmt.2014.11.010.

[20] E. Zorn *et al.*, 'Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease', *Blood*, vol. 106, no. 8, pp. 2903–2911, 2005, doi: 10.1182/BLOOD-2005-03-1257.

[21] A. De Masson *et al.*, 'CD24(hi)CD27^{+} and plasmablast-like regulatory B cells in human chronic graft-versus-host disease', *Blood*, vol. 125, no. 11, pp. 1830–1839, 2015, doi: 10.1182/BLOOD-2014-09-599159.

[22] E. Forcade *et al.*, 'An activated Th17-prone T cell subset involved in chronic graft-versus-host disease sensitive to pharmacological inhibition', *JCI Insight*, vol. 2, no. 12, Jun. 2017, doi: 10.1172/JCI.INSIGHT.92111.

[23] A. Kariminia *et al.*, 'Heterogeneity of chronic graft-versus-host disease biomarkers: association with CXCL10 and CXCR3+ NK cells', *Blood*, vol. 127, no. 24, pp. 3082–3091, Jun. 2016, doi: 10.1182/BLOOD-2015-09-668251.

[24] S. Paczesny *et al.*, 'National institutes of health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: III: The 2014 biomarker working group report', *Biology of Blood and Marrow Transplantation*, vol. 21, no. 5, pp. 780–792, May 2015, doi: 10.1016/j.bbmt.2015.01.003.

[25] M. C. Ciferri, R. Quarto, and R. Tasso, 'Extracellular Vesicles as Biomarkers and Therapeutic Tools: From Pre-Clinical to Clinical Applications', *Biology (Basel)*, vol. 10, no. 5, p. 359, May 2021, doi: 10.3390/BIOLOGY10050359.

[26] R. Baur *et al.*, 'Accumulation of T-cell-suppressive PD-L1high extracellular vesicles is associated with GvHD and might impact GvL efficacy', *J Immunother Cancer*, vol. 11, no. 3, p. 6362, Mar. 2023, doi: 10.1136/JITC-2022-006362.

[27] G. Lia *et al.*, 'Extracellular vesicles as potential biomarkers of acute graft-vs-host disease', *Leukemia*, vol. 32, no. 3, pp. 765–773, Mar. 2018, doi: 10.1038/LEU.2017.277;TECHMETA.

[28] G. Lia *et al.*, 'Extracellular Vesicles as Biomarkers of Acute Graft-vs.-Host Disease After Haploidentical Stem Cell Transplantation and Post-Transplant Cyclophosphamide', *Front Immunol*, vol. 12, p. 816231, Jan. 2022, doi: 10.3389/FIMMU.2021.816231/BIBTEX.

[29] M. J. Hartwell *et al.*, 'An early-biomarker algorithm predicts lethal graft-versus-host disease and survival', *JCI Insight*, vol. 2, no. 3, Jul. 2018, doi: 10.1172/JCI.INSIGHT.89798.

[30] G. Storci *et al.*, 'Pre-transplant CD69+ extracellular vesicles are negatively correlated with active ATLG serum levels and associate with the onset of GVHD in allogeneic HSCT patients', *Front Immunol*, vol. 13, p. 1058739, Jan. 2023, doi: 10.3389/FIMMU.2022.1058739/FULL.

[31] H. K. Srinagesh *et al.*, 'The MAGIC algorithm probability is a validated response biomarker of treatment of acute graft-versus-host disease', *Blood Adv*, vol. 3, no. 23, pp. 4034–4042, 2019, doi: 10.1182/BLOODADVANCES.2019000791.

[32] Q. Song *et al.*, 'The link between tissue-resident memory T cells and graft-versus-host disease', *Blood&Genomics, 2022, Vol. 6, Issue 2, Pages: 81-90*, vol. 6, no. 2, pp. 81–90, Dec. 2022, doi: 10.46701/BG.2022022022026.

[33] S. J. Divito *et al.*, 'Peripheral host T cells survive hematopoietic stem cell transplantation and promote graft-versus-host disease', *J Clin Invest*, vol. 130, no. 9, pp. 4624–4636, Sep. 2020, doi: 10.1172/JCI129965.

[34] G. P. de Almeida *et al.*, 'Human skin-resident host T cells can persist long term after allogeneic stem cell transplantation and maintain recirculation potential', *Sci Immunol*, vol. 7, no. 67, Jan. 2022, doi: 10.1126/SCIIMMUNOL.ABE2634.

[35] J. Strobl *et al.*, 'Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation', *J Exp Med*, vol. 218, no. 11, Nov. 2021, doi: 10.1084/JEM.20210417.

[36] D. Cibrián and F. Sánchez-Madrid, 'CD69: from activation marker to metabolic gatekeeper', *Eur J Immunol*, vol. 47, no. 6, pp. 946–953, Jun. 2017, doi: 10.1002/EJI.201646837.

[37] J. Smolders *et al.*, 'Tissue-resident memory T cells populate the human brain', *Nat Commun*, vol. 9, no. 1, Dec. 2018, doi: 10.1038/S41467-018-07053-9.

[38] T. Sathaliyawala *et al.*, 'Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets', *Immunity*, vol. 38, no. 1, pp. 187–197, Jan. 2013, doi: 10.1016/j.immuni.2012.09.020.

[39] J. J. C. Thome and D. L. Farber, 'Emerging concepts in tissue-resident T cells: Lessons from humans', *Trends Immunol*, vol. 36, no. 7, pp. 428–435, Jul. 2015, doi: 10.1016/j.it.2015.05.003.

[40] M. Yáñez-Mó *et al.*, 'Biological properties of extracellular vesicles and their physiological functions', *J Extracell Vesicles*, vol. 4, no. 2015, pp. 1–60, 2015, doi: 10.3402/JEV.V4.27066.

[41] A. Bacigalupo *et al.*, 'Unmanipulated haploidentical bone marrow transplantation and post-transplant cyclophosphamide for hematologic malignanices following a myeloablative conditioning: an update', *Bone Marrow Transplant*, vol. 50 Suppl 2, pp. S37–S39, Jun. 2015, doi: 10.1038/BMT.2015.93.

[42] L. Luznik *et al.*, 'HLA-Haploidentical Bone Marrow Transplantation for Hematologic Malignancies Using Nonmyeloablative Conditioning and High-Dose, Posttransplantation Cyclophosphamide', *Biology of Blood and Marrow Transplantation*, vol. 14, no. 6, pp. 641–650, Jun. 2008, doi: 10.1016/j.bbmt.2008.03.005.

[43] F. Bonifazi *et al.*, 'Low-Dose Anti-T Lymphoglobulin as Prophylaxis for Graft-versus-Host Disease in Unrelated Donor Transplantations for Acute Leukemias and Myelodysplastic Syndromes', *Biology of Blood and Marrow Transplantation*, vol. 24, no. 12, pp. 2450–2458, Dec. 2018, doi: 10.1016/j.bbmt.2018.07.011.

[44] N. Koliha *et al.*, 'A novel multiplex bead-based platform highlights the diversity of extracellular vesicles', *J Extracell Vesicles*, vol. 5, no. 1, Jan. 2016, doi: 10.3402/JEV.V5.29975.

[45] O. P. B. Wiklander *et al.*, 'Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures', *Front Immunol*, vol. 9, no. JUN, Jun. 2018, doi: 10.3389/FIMMU.2018.01326.

[46] A. Möller and R. J. Lobb, 'The evolving translational potential of small extracellular vesicles in cancer', *Nat Rev Cancer*, vol. 20, no. 12, pp. 697–709, Dec. 2020, doi: 10.1038/S41568-020-00299-W.

[47] M. Marchisio *et al.*, 'Flow Cytometry Analysis of Circulating Extracellular Vesicle Subtypes from Fresh Peripheral Blood Samples', *Int J Mol Sci*, vol. 22, no. 1, p. 48, Jan. 2020, doi: 10.3390/IJMS22010048.

[48] R. P. McNamara *et al.*, 'Imaging of surface microdomains on individual extracellular vesicles in 3-D', *J Extracell Vesicles*, vol. 11, no. 3, Mar. 2022, doi: 10.1002/JEV2.12191.

[49] B. V. Kumar *et al.*, 'Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites', *Cell Rep*, vol. 20, no. 12, pp. 2921–2934, Sep. 2017, doi: 10.1016/j.celrep.2017.08.078.

[50] J. M. Schenkel and D. Masopust, 'Tissue-resident memory T cells', *Immunity*, vol. 41, no. 6, pp. 886–897, Dec. 2014, doi: 10.1016/j.immuni.2014.12.007.

[51] L. R. Shiow *et al.*, 'CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs', *Nature*, vol. 440, no. 7083, pp. 540–544, Mar. 2006, doi: 10.1038/NATURE04606.

[52] R. Greco *et al.*, 'Interleukin-6 as Biomarker for Acute GvHD and Survival After Allogeneic Transplant With Post-transplant Cyclophosphamide', *Front Immunol*, vol. 10, p. 2319, Oct. 2019, doi: 10.3389/FIMMU.2019.02319.

[53] R. G. Resende *et al.*, 'Investigation of functional IL-10 gene polymorphism and IL-10 levels in acute graft-versus-host disease', *J Clin Immunol*, vol. 30, no. 3, pp. 465–473, May 2010, doi: 10.1007/S10875-010-9377-6.

[54] N. Giesen *et al.*, 'CXCL9 Predicts Severity at the Onset of Chronic Graft-versus-host Disease', *Transplantation*, vol. 104, no. 11, pp. 2354–2359, Nov. 2020, doi: 10.1097/TP.0000000000003108.

[55] M. Guo and C. Zhu, 'Serum neurofilament light chain, markers of systemic inflammation and clinically relevant depressive symptoms in US adults', *J Affect Disord*, vol. 363, pp. 572–578, Oct. 2024, doi: 10.1016/j.jad.2024.07.146.

[56] V. Tkachev *et al.*, 'Spatiotemporal single-cell profiling reveals that invasive and tissue-resident donor CD8+ memory T cells drive gastrointestinal acute graft-versus-host disease', *Sci Transl Med*, vol. 13, no. 576, p. eabc0227, Jan. 2021, doi: 10.1126/SCITRANSLMED.ABC0227.

[57] S. Corgnac, M. Boutet, M. Kfoury, C. Naltet, and F. Mami-Chouaib, 'The Emerging Role of CD8+ Tissue Resident Memory T (TRM) Cells in Antitumor Immunity: A Unique Functional Contribution of the CD103 Integrin', *Front Immunol*, vol. 9, no. AUG, Aug. 2018, doi: 10.3389/FIMMU.2018.01904.

[58] J. Y. Heo and D. S. Im, 'Pro-Inflammatory Role of S1P3 in Macrophages', *Biomol Ther (Seoul)*, vol. 27, no. 4, pp. 373–380, 2019, doi: 10.4062/BIOMOLTHER.2018.215.

[59] L. Wang and V. W. Y. Lui, 'Emerging Roles of ALK in Immunity and Insights for Immunotherapy', *Cancers (Basel)*, vol. 12, no. 2, Feb. 2020, doi: 10.3390/CANCERS12020426.

[60] J. L. Ferrara, J. E. Levine, P. Reddy, and E. Holler, 'Graft-versus-host disease', *The Lancet*, vol. 373, no. 9674, pp. 1550–1561, 2009, doi: 10.1016/S0140-6736(09)60237-3.

[61] S. W. Choi *et al.*, 'Change in plasma tumor necrosis factor receptor 1 levels in the first week after myeloablative allogeneic transplantation correlates with severity and incidence of GVHD and survival', *Blood*, vol. 112, no. 4, pp. 1539–1542, Aug. 2008, doi: 10.1182/BLOOD-2008-02-138867.

[62] M. L. Dustin, 'Role of adhesion molecules in activation signaling in T lymphocytes', *J Clin Immunol*, vol. 21, no. 4, pp. 258–263, 2001, doi: 10.1023/A:1010927208180/METRICS.

[63] K. L. Csencsits and D. K. Bishop, 'Contrasting Alloreactive CD4+ and CD8+ T Cells: There's More to It Than MHC Restriction', *American Journal of Transplantation*, vol. 3, no. 2, pp. 107–115, Feb. 2003, doi: 10.1034/J.1600-6143.2003.00036.X.

[64] G. R. Hill *et al.*, 'The p55 TNF-alpha receptor plays a critical role in T cell alloreactivity', *J Immunol*, vol. 164, no. 2, pp. 656–663, Jan. 2000, doi: 10.4049/JIMMUNOL.164.2.656.