

Hematogones as a Prognostic Indicator in Allogeneic Hematopoietic Stem Cell Transplantation in Severe Aplastic Anemia, Single Center Experience

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ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the treatment of choice for severe aplastic anemia (SAA) in young age. Hematogones (HG), reflect regeneration of bone marrow post-chemotherapy as well as post-HSCT. The aim of this study is to examine the impact of HGs in comparison to other biomarkers (pre- transplant ferritin and the count of infused CD34⁺ cells) on allo-HSCT in SAA. We studied 21 SAA patients, treated with allo-HSCT from MSD (matched sibling donor). HG were measured at engraftment (n=18) by using CD34, CD38, CD10, CD19 antibodies. Cyclosporine with either Methotrexate (CSA+MTX, n=12) or Methylprednisolone (CSA+MP; n=9) used for graft versus host (GVHD) prophylaxis. All patients were followed up for GVHD, infections, counts and overall survival (OS). We used Receiver- Operator Characteristics curve (ROC) to determine HG cut off; 0.37% of total mononuclear cells (MNC), CD34⁺ cut off; 6.8cell x10⁶/cells/Kg and ferritin cut off; 1337ng/dl. Patients with high HG had better total white cell count (WBC); *P*=.03, absolute neutrophil count (ANC); *P*=.04, platelet; *P*=.02 at engraftment time in addition to better OS (*P*=.005). On the other side CD34 and ferritin didn't impact OS. In conclusion we showed that HG at engraftment directly affect the outcome of patients with SAA treated with allo-HSCT.

Keywords: Hematogones, Severe Aplastic Anemia, Allogeneic Hematopoietic Stem Cell Transplant

INTRODUCTION

Acquired aplastic anemia is a hematological disorder where both pancytopenia and bone marrow hypoplasia exist [1], in the absence of marrow dysplasia, malignant infiltration or fibrosis with incidence 2.34/million [2]. SAA according to the international aplastic anemia study group defined as 2 out of 3 peripheral blood criteria (ANC <0.5 x 10⁹/L, platelet < 20 X 10⁹/L and reticulocyte <1% corrected) and bone marrow cellularity <30% [3]. Aplastic anemia as a serious hematologic disease with mortality rate in 2 years after diagnosis was over 40%; transplant conferred a better outcome [4].

Allo- HSCT is the standard of care in SAA patients younger than 40 and with available MSD, other than this Immunosuppressive therapy (IST) should be considered, mostly in the form of anti-thymocyte globulin (ATG) and cyclosporine (CSA) [1, 5-7]. Matched unrelated donor, haplo-identical and cord blood HSCT introduced new horizon of treatment of SAA without MSD with very comparable outcome and survival [5, 7-9].

Unfortunately very few studies were carried out in Egypt regards therapeutic options of SAA although allo-HSCT which is considered the standard line of treatment in young patients was used in SAA patients with available MSD [10]. Many prognostic indicators that affects allo-HSCT were previously discussed in literature such as age of recipient [11, 12], ferritin level [13], type of donor [11, 14], conditioning

regimen [15-17], source of stem cell, CD34⁺ cells count [18-20], and GVHD prophylaxis [21].

HG, normal B lymphocyte precursors that is present in bone marrow and contribute to humoral immunity [22-24]. Increased HG noticed in healthy children known as reactive HG [24], while regenerative HG exist in non –malignant disorders such as autoimmune diseases, congenital cytopenia, acquired immunodeficiency [25] and neoplastic disorder such as lymphoma [26]. In addition to regeneration of humoral immunity post chemotherapy and post allo-HSCT [23, 24, 27]. HG were considered as a positive prognostic indicator especially in allo-HSCT [27], cord blood transplantation [28] and post-acute myeloid leukemia (AML) treatment [29].

Early HGs have round nuclei with indentations, scant cytoplasm, and inconspicuous homogeneous chromatin with indistinct or at times variably prominent nucleoli. Presence of one or more nucleoli reflects HGs immaturity, cytoplasm is moderately to deeply basophilic and lacks inclusions, granules or vacuoles [30]. Using flow cytometer HG were classified to different phases CD10, CD19, CD20, CD22, CD34, and CD38 [28].

HG was studied in detection of minimal residual disease of acute lymphoblastic leukemia (ALL) [31], in addition to a prognostic

marker in hematological malignancies post chemotherapy [29] and post HSCT [32]. CD34⁺ cell dose another positive prognostic indicator especially in peripheral blood allo-HSCT in SAA due to rapid engraftment which lead to less treatment related mortality [18, 20].

In addition to ferritin level with different cut off levels [13, 33] and GVHD prophylaxis regimens either using MTX or MP with CSA used to impact the outcome of HSCT [34, 35]. The aim of our study is to detect the effect of HG as a prognostic biomarker in our SAA patients treated with allo-HSCT.

This is a prospective cross-sectional study, following institutional approval, 21 SAA patients with SAA were consented and enrolled. All patients were treated with MSD allo- HSCT, between December 2013 and January 2016. Patient's inclusion criteria; SAA patients, younger than 40, normal cytogenetic, had HLA matched sibling donor, assigned to undergo peripheral blood HSCT. While exclusion criteria; patients who were elder than 40 years, clinically unfit, had no HLA matched sibling donor, abnormal cytogenetic, diagnosed with inherited bone marrow failure or paroxysmal nocturnal hemoglobinuria (PNH) (Figure1).

PATIENTS AND METHODS

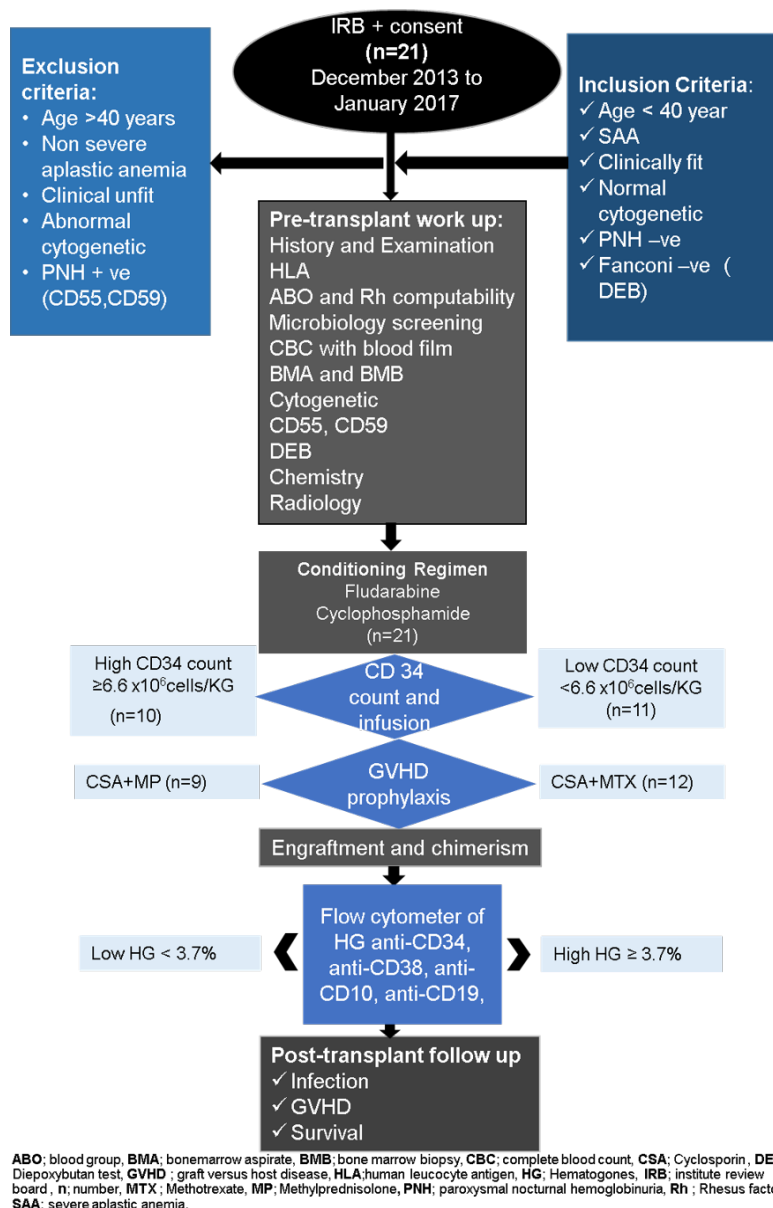


Figure 1: A Diagrammatic Flow Chart: Shows Logistics and Steps Performed for Patients throughout this Study. Inclusion, Exclusion Conditions, Pre-Transplant Work up for Every Patient Enrolled in this Study are Detailed. In Addition to Check List of Follow up Post HSCT Transplant, Survival was Used as an Overall Successful Outcome

We examined patients and donors for medical history, clinical examination, pre- transplant intermediate-resolution HLA typing by polymerase chain reaction sequence- specific oligonucleotides probe (PCR-SSO) for HLA class-I (HLA-A, -B, -C) and class-II (HLA-DRB1, -DQB1) [36], blood (ABO & Rh) grouping, microbiology screening

(HBsAg, HBsAb, HBeAb, HCV-Ab, HCV-PCR, HIV-Ab, CMV IgM / IgG, Toxoplasma IgG / IgM, CBC with blood film, examination of bone marrow aspirate and biopsy, immunophenotyping to exclude PNH (CD55 & CD59; patients only), cytogenetic study, diepoxybutan test to exclude Fanconi anemia, and metabolic profile which included

measurements of aspartate transaminase, alanine transaminase, total bilirubin, alkaline phosphatase, urea, creatinine, uric acid, albumin, calcium, random blood sugar, sodium, potassium and serum ferritin [37, 38]. We examined patients only for abnormal radiological findings using echocardiography, abdominal & pelvic sonar, sinuo-pulmonary CT scanning and dental consultation to make sure that patient is free from any septic focus and is clinically fit, before allo-HSCT procedure [37].

Fludarabine 30 mg/m² I.V. from day -6 to -2, cyclophosphamide 60 mg/kg from day -3 to -2 (FLU/CYC) used as conditioning regimen in all patients before HSCT [38]. On day zero, stem cells were harvested from donor peripheral blood after injecting granulocyte colony stimulating factor (G-CSF) for 5 consecutive days; 10 mcg/kg/day [38] through 1-2 apheresis cycles on COBE Spectra continuous cell separator (Gambro, Lakewood, CO, USA) using Spin-Nebraska protocol [39]. Once confirmed total CD34⁺ count ($\geq 3 \times 10^9$ /L), infused to patient's central line immediately following apheresis procedure complete (day 0). GVHD prophylaxis was CSA 3 mg/kg body weight as I.V. from day -1 in combination with MTX 15 mg/m² I.V. on day +1, and 10 mg/m² I.V. on days +3, +6 and +11 respectively, with leucovorin rescue in 12 patients while other 9 patients received cyclosporine 3 mg/kg bodyweight as I.V. continuous infusion from day

-1 in combination with MP 2 mg/Kg start from day +4 in a trial to improve patients outcome and decrease post-transplant immunological complications [40]. At the time of discharge all patients were switched to antibacterial, antifungal, antiviral and GVHD prophylaxis.

Successful engraftment is defined when absolute neutrophils count exceed 0.5×10^9 /L for 3 consecutive days [40]. Then analysis of donor/ recipient cell chimerism, PCR amplification of polymorphic short tandem repeats (STR) performed to confirm engraftment of donor cells [41].

Flow Cytometer HG Detection

Bone marrow aspirate obtained from 21 patients on engraftment day; 3 samples failed in processing and preparation while 18 samples we could detect the HG levels in them. Cells were stained with anti-CD34 (FL4), anti-CD38 (FL1), anti- CD10 (FL2), anti- CD19 (FL3), and lineage (Lin) mixture (anti-CD3, -CD4, -CD8 -CD20, -CD14, and - CD56, Supplementary Table 1). Early HGs referred to certain cell population both CD38⁺CD10⁺ CD19⁺CD34⁺ and CD38⁺CD10⁺CD19⁺CD34⁻. Late HGs referred to cell population CD38⁺CD10⁺CD19⁺CD34⁻, and CD38⁺CD10⁺CD19⁺CD34⁻ [23, 24, 28, 42, 43].

SUPPLEMENTARY Table 1: Monoclonal Antibodies used for Immunophenotyping of Hematopoietic Stem Cells Detection

Monoclonal Antibodies	Fluorochrome	Clone	Monoclonal Antibodies	Fluorochrome	Clone
CD38*	FITC	HIT2	CD10*	PE	MEM-78
CD8*	FITC	SK1	CD19*	PE CF594	HIB19
CD3*	FITC	UCHT1	CD34**	PE-PC7	581
CD4*	FITC	RPA-T4	CD20	FITC	2H7
CD14*	FITC	MEM-15	CD56*	FITC	MEM 188

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Samples were run on multicolor flow cytometry (Coulter, Navios) with an integrated intelligent analysis algorithm, which auto-sets gates, regions and compensation to flag samples that don't meet auto-validation criteria or require operator review. The prism processor represents data from 2, 3, or 4 Ab combinations as 4, 8 or 16 evenly spaced peaks in single-parameter graph. Each peak represents an Ab combination or phenotype. For example, in this four-color experiment, the first peak represents the cells negative for all four parameters, 2nd peak represents cells that are positive to the first parameter and negative for all other parameters and so on; with the last peak representing cells positive for the four parameters. The height of the peak is proportional to the number of events belonging to the phenotype represented by the peak. To use prism, a positive range must first be selected for each parameter. Whenever an event is detected, the prism processor determines if the event lies within the positive range for any of the parameters and increments the appropriate channel. Prism provides a simple way of obtaining quantitative results from complex multicolor data. However, there is no indication of mean fluorescence channel or SD for each parameter in prism alone.

All patient followed up in outpatient clinic for infections; single oral temperature of 38.3° C (101° F) or a temperature of greater than 38.0° C (100.4° F) sustained for more than 1 hour (followed by culture and sinus pulmonary CT), acute GVHD and chronic GVHD [44] and OS.

Statistical Analysis

The categorical variables were compared with Fisher exact test, continues variables were compared using unpaired t-test or Kruskal-

Wallis test with the calculation of confidence interval (CI) and paired t-test was used to compare pre- vs. post-transplant variables for same patients' data. The probability of OS was estimated according to Kaplan-Meier method and compared with Log-rank test. Receiver operating Characteristic (ROC) curve analysis was used to examine for best cutoff biomarker values with sensitivity, specificity, likelihood ratio (LR), and area under the curve. *P* value less than .05 was considered as significant. All statistical analysis and graph presentation were done by the Graph Pad Prism software version 7.00 for Windows, Graph Pad Software, La Jolla California USA, www.graphpad.com.

Results

Clinical Characteristics of the Study Cohort and the Auto-HSCT Outcome

Our cohort included 21 patients with clinical characteristics described in Table 1. The mean $\pm$ SD of age; 19 ± 6.4 years. Males were 81 % (n=17) of patients. Body surface area means $\pm$ SD was 1.6 ± 0.4 . Blood group A was found in 43% (n=9) patients while AB was only in one patient (5%); In Viral screening; one patient had positive HBV surface antigen positive while 90% (n=19) had IgG positive CMV and 2 (10%) had positive IgM, Toxoplasmosis was positive IgG in 24% of patients and only 1 patient had IgM positive. Pre-transplant CBC, median (range), WBC was 2.2×10^9 /L (0.7-4.3), ANC; 0.5×10^9 /L (0.1-1.2), HB 7.3 g/dl (3.8-13.9), platelet 8×10^9 /L (3-15), bone marrow cellularity 20 (5-40%) and serum ferritin 1101 ng/ml (397-3959).

Table 1: Clinical Characteristics of the Study Cohort (n=21)

	Recipient	Donor
Male n (%)	17 (81%)	13 (62%)
Age (mean± SD)	19 ± 6.4	21 ± 10.2
BSA (mean± SD)	1.6 ± 0.4	1.5 ± 0.4
Screening n (%)		
A	9 (43%)	10 (48%)
B	4 (19%)	3 (14%)
AB	1 (5%)	1 (5%)
O	7 (33%)	7 (33%)
Hepatitis B (HBsAg)	1 (5%)	0
CMV (IgG)	19 (90%)	19 (90%)
CMV (IgM)	2 (10%)	2 (10%)
Toxoplasma (IgG)	5 (24%)	3 (14%)
Toxoplasma (IgM)	1 (5%)	0
Pre-transplant labs median (range)		
WBC x 10 ⁹ /L	2.2 (0.7-4.3)	
ANC x 10 ⁹ /L	0.5 (0.1-1.2)	
HB g/dl	7.3 (3.8-13.9)	
Platelet x 10 ⁹ /L	8 (3-15)	
Ferritin ng/mL	1101 (397-3959)	
BM Cellularity n (%)	20 (5-40%)	
Conditioning n (%)		
FLU/CYC	21 (100%)	
GVHD Prophylaxis n (%)		
CSA±MTX	12 (57%)	
CSA/MP	9 (43%)	

ANC, Absolute Neutrophil Count, BM, Bone Marrow, BSA, Body Surface Area, CMV, Cytomegalovirus, CSA, Cyclosporine, CYC, Cyclophosphamide, FLU, Fludarabine, HB, Hemoglobin, GVHD, Graft Versus Host Disease, MP, Methylprednisolone, MTX, Methotrexate, SD, Standard Deviation, WBC, White Blood Cells.

Donors' characteristics; 62% (n=13) of donors were males, with age mean ± SD; 21±10.2 years and body surface area 1.5±0.4. Regards blood group 48% (n=10) had blood group A and one patient had blood group AB, thus leads to three patients (14%) had major ABO major incompatibility. All donors were negative for HBV surface antigen while 90% had IgG positive CMV, 10% had IgM CMV positive and 14% had IgG toxoplasmosis positive with no donors with IgM toxoplasmosis positive.

The median of engraftment day was day 19 with range (day 15-27), all patients were successfully engrafted. CBC median (range) at

engraftment; WBC 4.2 x 10⁹ /L (1.3-8.6), ANC 1.6 x 10⁹ /L (0.5-3), HB 9.4 x g/dl (8.2-10) and platelet 63 x 10⁹ /L (15-156); Table 2. In post-transplant follow up 8 (38%) of patients had acute GVHD; half of which had acute GVHD grade 0-1 and half had acute GVHD grade 2-4, while three patients had chronic GVHD. One patient develops de-novo chronic GVHD and 2 continued to have chronic GVHD. The incidence of infection was 67% in median timing day 5 through 47 months follow up (Supplementary Table 2). Median number of packed red blood cells transfusions was 4 units and platelet transfusion 3 times of 6 units.

Supplementary Table 2: Individualized Patient in our Cohort with GVHD and Infection Outcome

Patient No.	Age	Gender	aGVHD 0-1	aGVHD 2-4	Denovo cGVHD	2 nd cGVHD	cGVHD Extension	Infection
1	21	M	0	0	0	0	0	1
2	24	M	0	0	0	0	0	1
3	11	F	0	0	0	0	0	0
4	9	M	0	0	Skin, MM	Skin	Limited	0
5	23	M	0	0	0	0	0	1
6	21	M	0	0	0	0	0	1
7	22	F	1 GIT	0	0	0	0	1
8	15	M	0	0	0	0	0	1
9	12	F	1 GIT	0	0	0	0	1
10	18	M	0	G3 GIT	0	0	0	0
11	18	M	0	0	0	0	0	1
12	27	M	MM, GIT	0	0	0	0	1
13	7	M	0	GIT	0	0	0	1
14	17	M	0	0	0	0	0	1
15	19	F	0	0	0	0	0	1
16	30	M	0	G3, GIT	0	1	limited	0
17	20	M	0	0	0	0	0	1
18	33	M	0	0	0	0	0	1
19	17	M	1 MM	0	0	0	0	0
20	18	M	0	0	0	0	0	0
21	19	M	0	G2 Skin	0	Skin	Limited	0

aGVHD, Acute Graft Versus Host Disease, cGVHD, Chronic Graft Versus Host Disease, F, Female, GIT, Gastrointestinal Tract, M, Male, MM, Mucous Membrane.

Table 2: Patients HSCT Outcome

Engraftment	Lab's Median (Range)
Engraftment day	19(15-27)
WBC x 10 ⁹ /L	4.2 (1.3-8.6)
ANC x 10 ⁹ /L	1.6 (0.5-3)
HB g/dl,	9.4 (8.2-10)
Platelet x 10 ⁹ /L	63 (15-156)
GVHD n (%)	
aGVHD/0-1	4 (19%)
aGVHD/2-4	4 (19%)
De novo GVHD	1 (5%)
2nd GVHD	2(9.5%)
Infection	14 (67%)
Day Median (range)	5 (-3-13)
Line 1 (TZP/ Gen/ Amph) n (%)	10 (48%)
Line 2 (Ipm / Vori) n (%)	4 (19%)
Transfusion Support Median (Range)	
RBC Transfusion	4(0-7)
Platelet Transfusion	3(0-24)
Median Follow Up	47

Amph; Amphotericin, ANC, Absolute Neutrophil Count, Gentamycin, a/GVHD, acute/ Graft Versus Host Disease, HB, Hemoglobin, Imp, Imipenem, MP, Methylprednisolone, MTX, Methotrexate, TZP, Tazobactam, Vori, Voriconazole, WBC, White Blood Cells.

The mean $\pm$ SD of early HG was 0.8 ± 1.6 and median with range was 0.05(0-7.8) while mean $\pm$ SD of late HG was 0.6 ± 0.5 ($p=0.4$) and median with range 0.4(0-1.4).

Total HG mean $\pm$ SD was 0.7 ± 1.2 and median with range 0.2(0-7.8). As expected, mature B lymphocytes were less with mean $\pm$ SD; 0.05 ± 0.08 and median with range 0(0- 0.2) Supplementary Table 3.

Individualized HG measurement in each patient shown in Supplementary Figure 1. The mean $\pm$ SD of CD34 cells count infused to recipients on day zero after G-CSF mobilization and one day apheresis collection was $7.2 \pm 3.4 \times 10^6$ /L, median was 7×10^6 /L and range (3-15.8) $\times 10^6$ /L. While pre-transplant ferritin level mean $\pm$ SD was 1278 ± 623 ng/dl, median 1083 ng/dl and range (397-3305) ng/dl Supplementary Table 4.

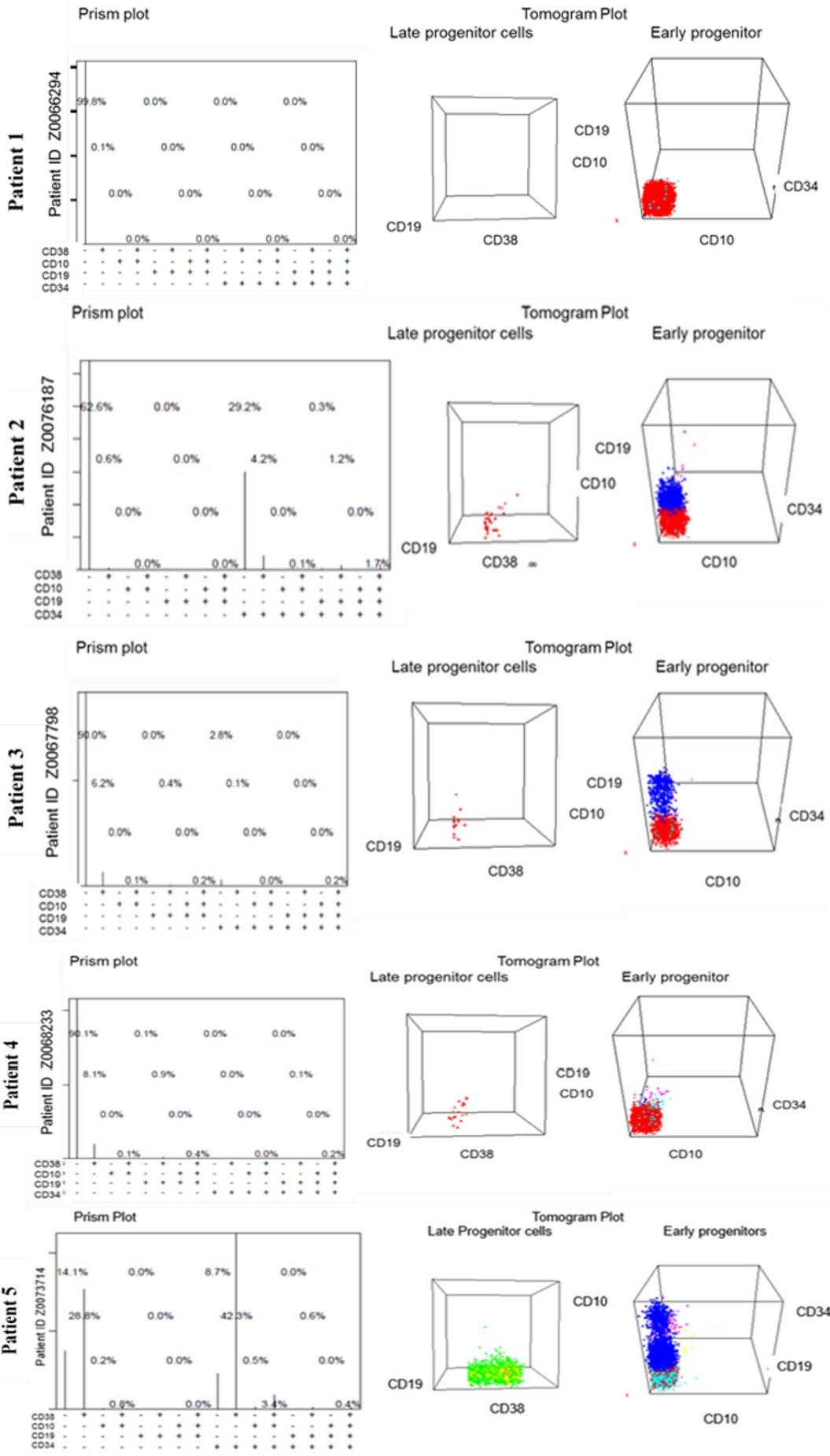
SUPPLEMENTARY Table 3: Flow Cytometer Measurement of Hematogones

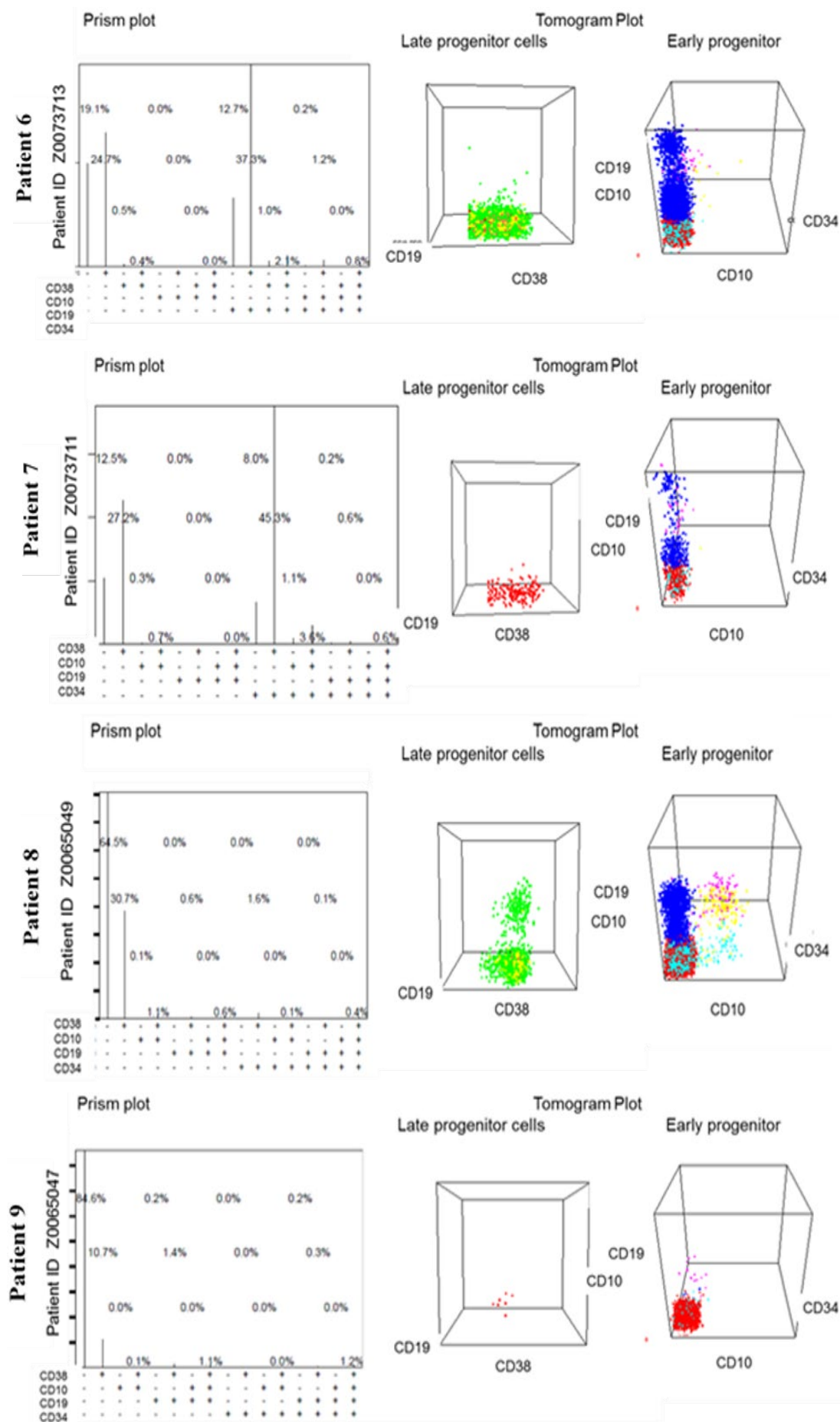
Flow Cytometer Marker	Mean $\pm$ SD	Median (Range)
Early HGs		
CD38 ⁻ CD10 ⁺ CD19 ⁻ CD34 ⁻	1.1 ± 2.1	0.25(0-7.8)
CD38 ⁻ CD10 ⁺ CD19 ⁻ CD34 ⁺	0.5 ± 1.1	0(0.4-1)
Total Early HGs	0.8 ± 1.6	0.05(0-7.8)
Late HGs		
CD38 ⁺ CD10 ⁺ CD19 ⁺ CD34 ⁻	0.5 ± 0.5	0.5(0-1.3)
CD38 ⁺ CD10 ⁻ CD19 ⁺ CD34 ⁻	0.6 ± 0.5	0.5(0-1.4)
Total Late HGs	0.6 ± 0.5	0.4(0-1.4)
Total HGs	0.7 ± 1.2	0.2(0-7.8)
Mature B	0.05 ± 0.08	0(0-0.2)

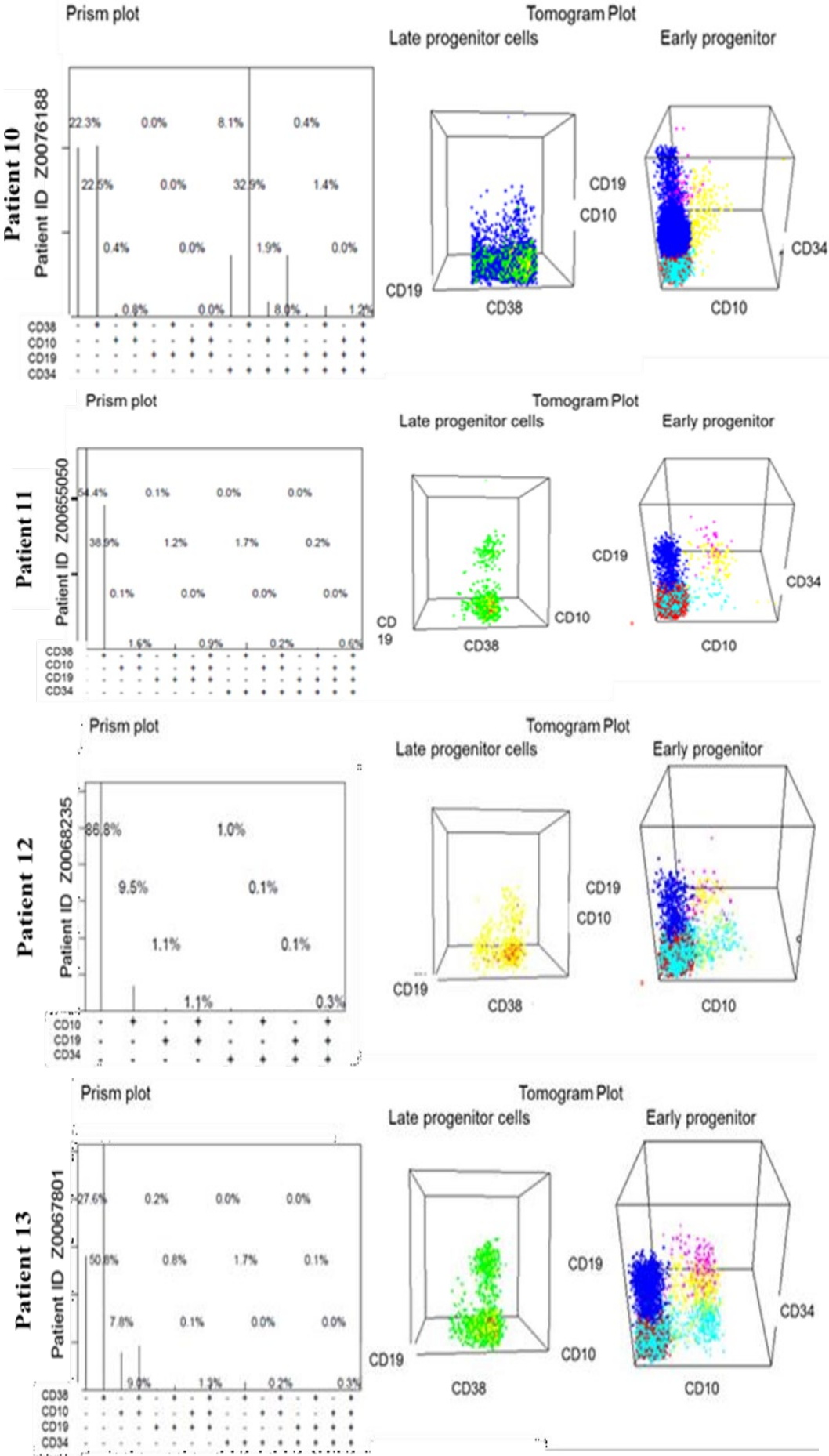
HGs, Hematogones, SD, Standard Deviation, + is Positive, - is Negative Sampling Day: 19 (15-27) n=18

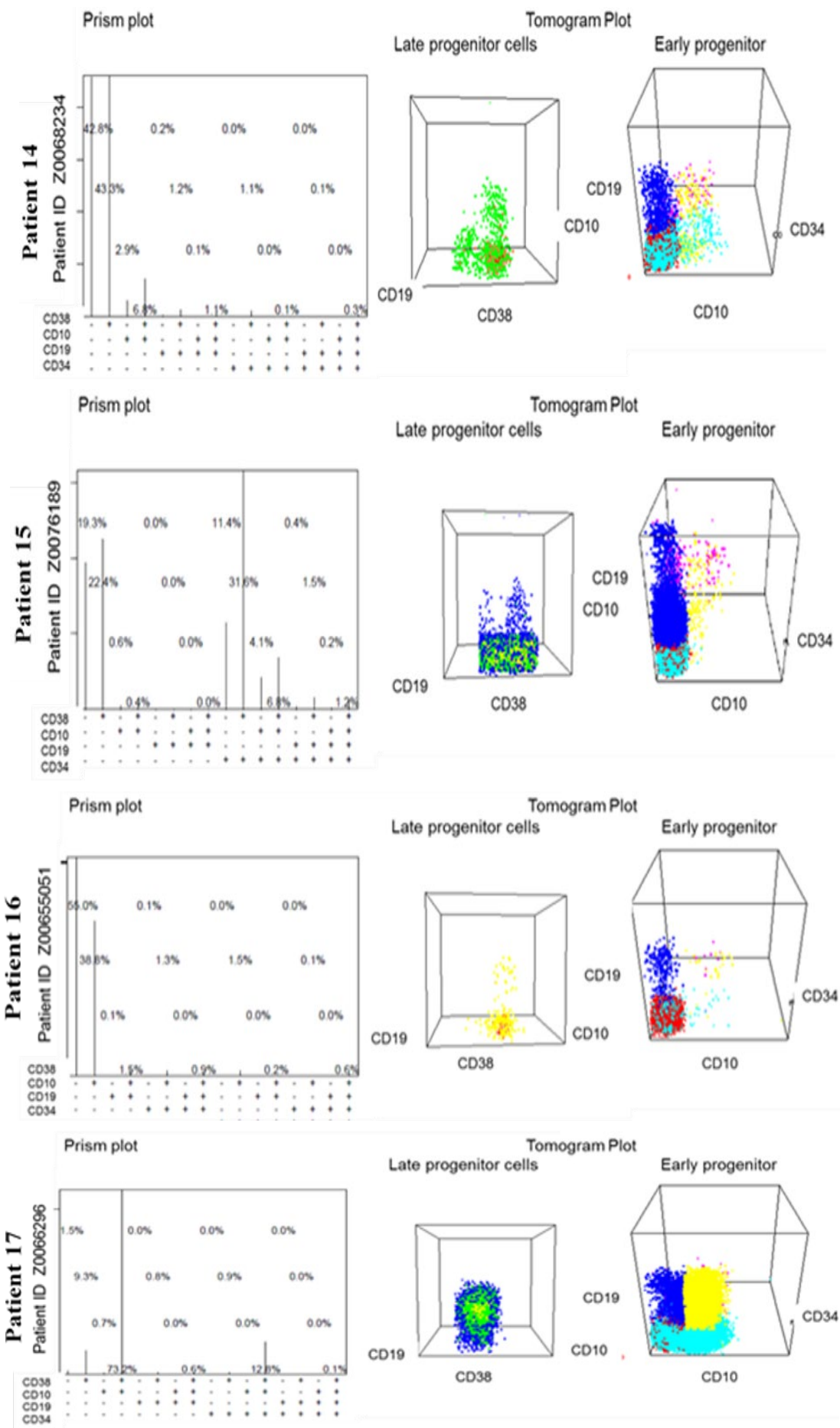
SUPPLEMENTARY Table 4: Individualized Prognostic Biomarkers Measurement

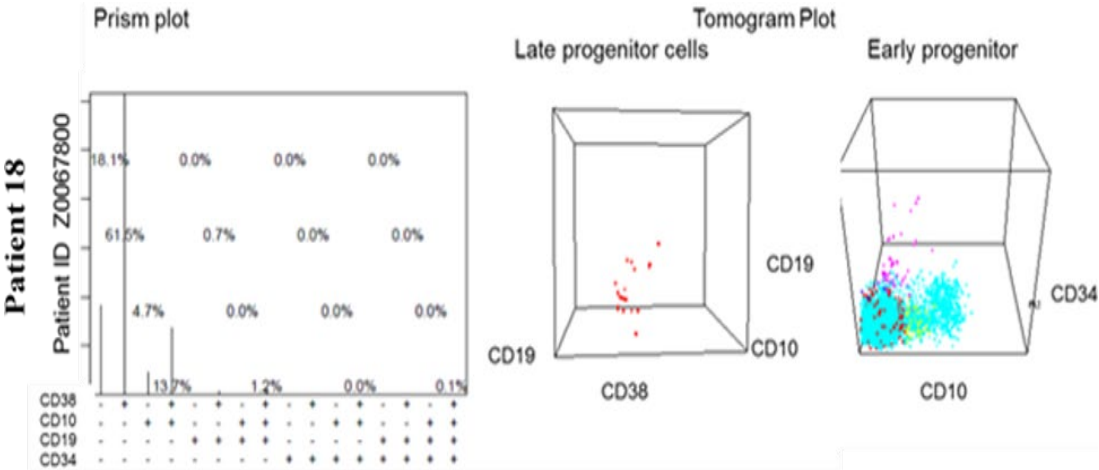
Number of Patients	Gender	HG %	CD 34 X10 ⁶ /L	Ferritin	Median Follow Up	Dead=1 / Alive=0
1	M	0.1	7.3	1101	8.2	1
2	F	0.1	7.2	1686	8.9	1
3	M	0.12	7	1500	11.9	1
4	M	0.23	8.9	1564	30	0
5	M	0.25	7	1300	25	0
6	F	0.32	7.5	675	22.7	0
7	M	0.35	3	1000	19.7	1
8	M	0.4	4.9	1373	36.6	0
9	M	0.43	3.5	920	20	0
10	M	0.52	14.5	780	36.6	1
11	M	0.63	15.8	1002	134	0
12	M	0.85	3.6	534	41.8	0
13	M	3.15	6.2	1861	37.2	0
14	F	2	6.1	1066	29.7	0
15	M	0.85	7	943	18.8	0
16	F	4.5	9.4	397	66.7	0
17	M	12.52	6.3	3305	12.6	0
18	M	3.38	4.2	2000	81.6	0
19	M	N/A	6.1	N/A	93.8	0
20	M	N/A	4.66	N/A	33.8	0
21	M	N/A	6.6	N/A	36.4	0







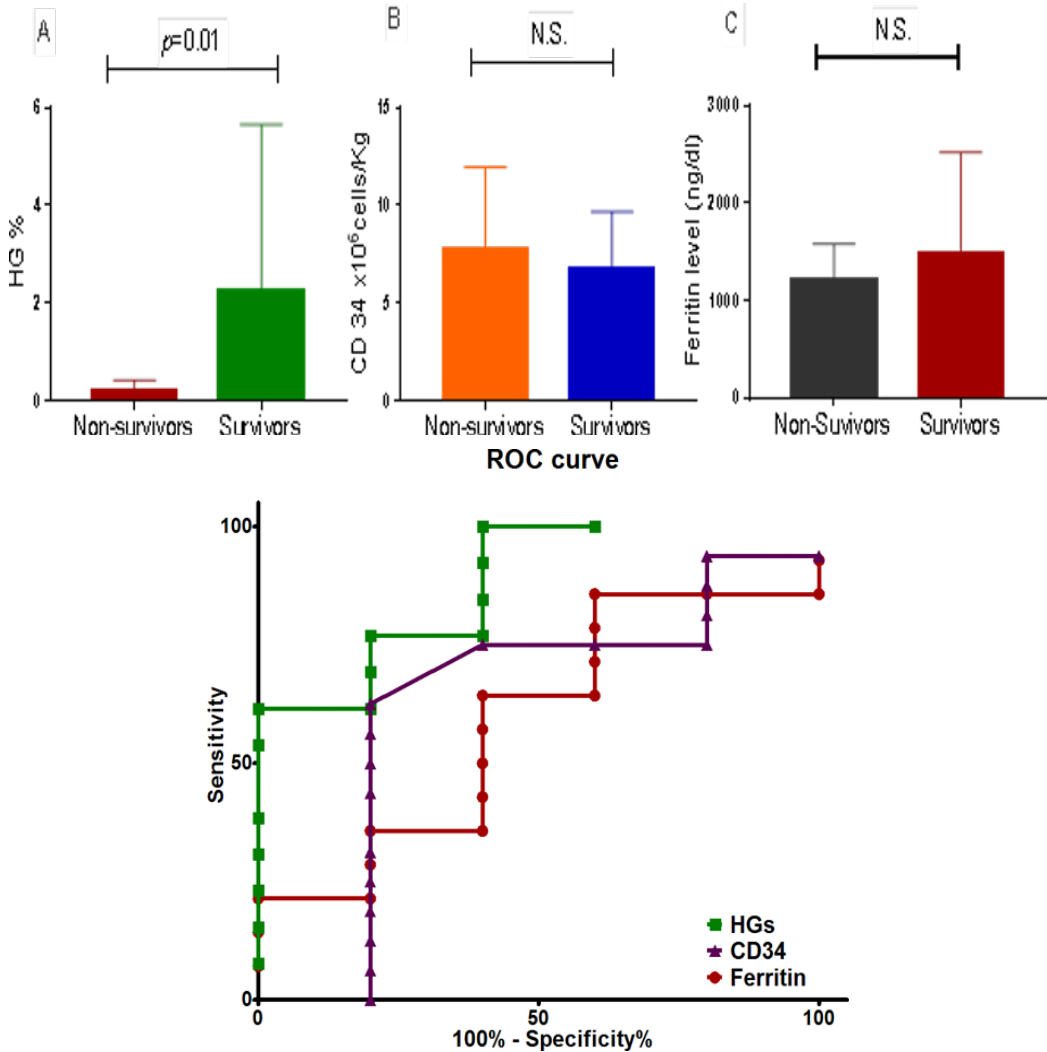




SUPPLEMENTARY FIGURE 1: Flow Cytometer Measurement of HGs. Left Panel Showed Prism Plot Describing Percent of CD38⁺, CD10⁺, and CD19⁺ and CD34⁺. Middle Panel Showed the late HGs Population and Right Panel Showed Early HGs Population. For Instance, Patient 1 and 2 Demonstrate Low HGs Levels While Patient 15 and 18 Demonstrate High HG Level

Interestingly the mean $\pm$ SD of HG levels of survivors (living) *vs.* non-survivors (2.3 ± 3.4 *vs.* 0.2 ± 0.2 ; Figure 2A) with statically significant difference $P=0.01$, CD34 count in survivors *vs.* non-survivors (6.7 ± 3 *vs.* 7.8 ± 4 ; Figure 2B) and ferritin level survivors *vs.* non-survivors (1493 ± 1025 *vs.* 1213 ± 371 ; Figure 2C). Using ROC curve analysis, Figure 2D, showed that HG best cut-off values of 0.37%, with AUC of 0.88, LR of 3.85, was the most sensitive 76.9% (95% CI;

35.4-84.8) and specific 80% (95% CI; 28.4-99.5) prognostic biomarker compared to CD34⁺ count with cut-off level; 6.8×10^6 cells/Kg, AUC; 0.63; LR; 3.13, sensitivity 62% (95% CI; 46.2-95) and specificity 80% (95% CI; 28.4-99.5), or ferritin with cut-off level; 1337, AUC; 0.59, LR; 1.6, sensitivity 64% with 95%CI; 35.14 - 87.24 and least specific 60% (95% CI;14.66-94.73).



	HG	CD34	Ferritin
Cut-off	0.37	6.8	1337
Sensitivity	76.9	62	62
Specificity	80	80	80
LR	3.85	3.13	1.61
AUC	0.88	0.63	0.59

Figure 2: Comparison between Outcomes with Different Biological Prognostic Indicators.; A) Mean $\pm$ SD of HG Levels of Survivors (living) ν s Non-Survivors with Statically Significant Difference $P=0.01$, B) CD34 Count in Survivors ν s Non-Survivors. C) Ferritin Level Survivors ν s Non-Survivors. Student T Test. D) ROC Curve: Compare the Three Prognostic Indicators Used; Showing HGs are the Most Sensitive and Specific. GVHD, Graft versus Host Disease, ROC, Receive Operator Curve

Hematogones Level as a Prognostic Biomarker Post Allogeneic HSCT in SAA

Although there was no significant difference regards the engraftment time in SAA patients treated with allo-HSCT in patients with high HG ($\geq 0.37\%$, $n=11$, median 19 days; range 15-23) ν s low HG subgroups ($<0.37\%$, $n=7$; median 17 days; range 15-22; $P=0.4$; Table 3). However, patients with high HG had better total WBC (median; 5×10^9 /L, range; 2-9 ν s 3.5, 1.3-5; 95% CI; -3.5 to -0.1, $P=0.02$), ANC (median; 2×10^9 /L, range; 0.8-4 ν s 1.4, 0.5-2; 95% CI, -1.5 to -0.02, $P=0.04$) and platelet (median; 90×10^9 /L, range; 21-156, ν s 32, 15-103, 95% CI; -89 to -6, $P=0.02$; Table 3) at engraftment time.

In addition to significant differences between pre- ν s post-transplant blood counts; mean $\pm$ SD, patients with high HG count; WBC (2.5 ± 1.1 ν s 5.1 ± 1.8 ; $p=0.007$), ANC (0.7 ± 0.4 ν s 2 ± 0.8 ; $p=0.0004$) and platelet ($26 \pm$ ν s 89 ± 46 ; $P=0.001$, Figure 3A). To a lesser extent, in patients' subgroup with low HG% below best cutoff value (3.7%), there was significant difference between pre-and post-transplant; ANC (0.6 ± 0.3 ν s 1.3 ± 0.5 ; $P=0.02$) and platelet (9 ± 4 ν s 41 ± 29 ; $P=0.3$, Figure 3A). There was no difference in incidence of acute and chronic GVHD, hospital acquired infections or transfusion frequency rate. Noticeably donor age of transplanted patients with high HG tended to be younger (Figure 3B), though not significant ($p>0.05$).

Table 3: Comparison between Low and High HGs Groups of Patients

	High HG ($>0.37\%$) (n=11)	Low HG ($<0.37\%$) (n=7)	95% CI
Age (Recipient) M $\pm$ SD	18.5 $\pm$ 7.2	20.5 $\pm$ 6.8	-5.1 to 9.3
Age (Donor) M $\pm$ SD	20.5 $\pm$ 11.7	22 $\pm$ 8.5	-9.2 to 12.5
Gender (Male) n (%)	9(82)	5(71)	
Sex in compatible (F to M) n (%)	2(18)	2(29)	
Pre-Transplant Median (Range)*			
WBC x 10^9 /L	2.7 (0.7-4.3)	2.1 (0.9-4.1)	-1.5 to 1.7
ANC x 10^9 /L	0.8 (0.1-1.2)	0.5 (0.3-1.2)	-0.5 to 0.2
HB g/dl	7.3 (3.8-13.9)	6.8(4.9-9.2)	-3.3 to 1.5
Platelet x 10^9 /L	8 (3-115)	8(4-16)	-45 to 10
BM cellularity	15% (0-30%)	25% (5-40%)	-9 to 23
Ferritin ng/mL	1002(397-3305)	1300(675-1686)	-738 to 682
CD34 cells x 10^6 cells/Kg Median (Range)*	6.2(3.5-15.8)	7.2(3-8.9)	-4.1 to 3
Engraftment Day Labs Median (Range)*			
Engraftment Day	19(15-23)	17(15-22)	-3.9 to 1.7
WBC x 10^9 /L	5(2-9)	3.5(1.3-5)	-3.5 to -0.1
ANC x 10^9 /L	2(0.8-4)	1.4((0.5-2)	-1.5 to -0.02
HB g/dl	9(8.3-10)	9.4(7.8-10)	-0.6 to 0.8
Platelet x 10^9 /L	90(21-156)	32(15-103)	-89 to -6
GVHD Prophylaxis n (%)			
CSA $\pm$ MTX	10(91)	1(14)	
CSA/MP	1(9)	6(86)	
Complication n (%)			
GVHD			
a GVHD/0-1	1(9)	2(28)	
a GVHD/2-4	2(18)	1(14)	
De novo GVHD	0	1(14)	
2nd GVHD	0	1(14)	
Infection n (%)	8(73)	6(86)	
Day Median (range)	5(1-13)	5(-3-19)	
Line 1 (TZP/ Gen/ Amph) n (%)	4(50)	4(57)	
Line 2 (Ipm / Vori) n (%)	4(50)	2(43)	
Transfusion Median (Range)			
RBC Transfusion	4(1-7)	2(0-6)	-3.5 to 0.7
Platelet Transfusion	2(2-6)	4(0-24)	-2 to 9.7
Median Follow Up (Range)	36 mo. (13-134)	20 mo. (9-30)	-57 to 0.3

a/GVHD, Acute/ Graft Versus Host Disease, ANC, Absolute Neutrophil Count, ATG, Antithymocyte Globulin, BM, Bone Marrow, BSA, Body Surface Area, CI, Confidence Interval, CSA, Cyclosporine, CMV, Cytomegalovirus, CYC, Cyclophosphamide, F, Female FLU, Fludarabine, GVHD, Graft Versus Host Disease, HB, Hemoglobin, HG, Hematogones, M $\pm$ SD, Mean $\pm$ Standard Deviation, Mo., Months, MP, Methylprednisolone, MMF, Mycophenolate Mofetil, MTX, Methotrexate, SAA, Severe Aplastic Anemia, TBI, Total Body Irradiation, WBC, White Blood Cells. * Range (Minimum-Maximum).

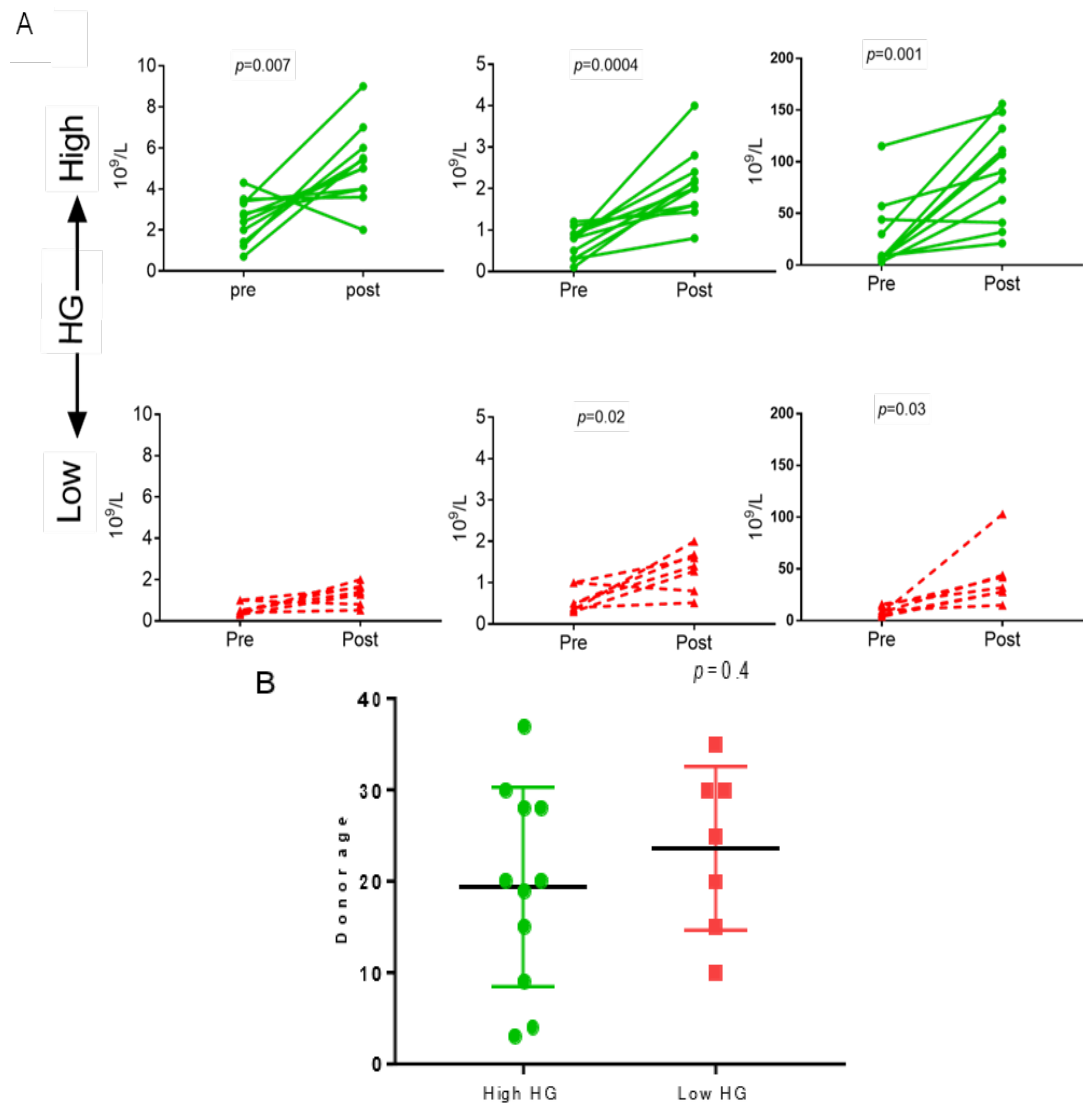


Figure 3: A) Differences in Pre and Post Transplantation Counts: Upper Panel; Mean +SD of Differences between Pre and Post Transplantation Counts in High HGs; WBC, ANC, HB and Platelet. Lower Panel; Mean +SD of Differences between Pre and Post Transplantation Counts in Low HG; WBC, ANC, HB and Platelet. B) Dot Plot Shows the Relation between Donor Age and HG Count. Inverse Proportion of Donor Age and HG Counts. Student T Test

CD34 Count as a Prognostic Indicator Post Allogeneic HSCT in SAA

Patients received higher CD34⁺ ($\geq 6.8 \times 10^6$ cells/Kg); determined using ROC curve in treatment of SAA by allogeneic HSCT had higher ANC and platelet count compared to patients who received lower CD34⁺ cells count; ANC (median; 1.5, range; 0.5-2.2 *vs* 2.1, 0.8-2.3, 95% CI; 0.0004 to 1.3, $P=0.02$) and platelet (median; 32, range; 15-111 *vs* 107, 42-156, 95%CI; -99 to -26, $P=0.02$; Table 4). Patients who received higher CD34⁺ count had significant difference between pre- vs post-transplant counts mean $\pm$ SD. WBC (2.4 ± 1.3 *vs* 5 ± 1.8 ; $P=0.007$), ANC (0.5 ± 0.3 *vs* 2 ± 0.6 ; $P=0.0001$), and platelet (12 ± 12

vs 45.5 ± 34 ; $P=0.02$, Figure 4) nevertheless, patients who received low CD34⁺ had significant rise in post-transplant ; WBC (2.3 ± 0.8 *vs* 3.7 ± 1.5 ; $P=0.01$), ANC (0.8 ± 0.3 *vs* 1.3 ± 0.7 ; $P=0.0009$) and platelet (30 ± 36 *vs* 108 ± 42 ; $P=0.0005$; Figure 4A).

In further analysis there was no statistically significant difference between HG % in patients with low and high CD34 count ($P=0.1$; Table 4).

In fact, the relation was inversely proportionate. In addition, there were no noticed differences in incidence of infection, acute and chronic GVHD.

Table 4 (a): Outcome According to CD34 Count

	Low CD34 (n=11)	High CD34 (n=10)	CI 95%
HG M $\pm$ SD	3 $\pm$ 4	0.7 $\pm$ 1.35	-0.5 to 5
Age M $\pm$ SD	21 $\pm$ 7	18 $\pm$ 5.5	-3 to 8.7
Gender (male) n (%)	8 (72)	9 (90)	
Pre-transplant Labs median (range)*			
WBC x $10^9/L$	2.3 (0.7-4.3)	2.2 (1.2-3.5)	-1 to 1
ANC x $10^9/L$	0.5 (0.3-1)	0.8 (0.5-1.2)	-0.5 to 0.02
HB g/dl	7.2 (3.8-8.5)	9.2 (5.4-13.9)	-4.3 to -0.4
Platelet x $10^9/L$	8 (4-44)	14 (3-115)	-43.3 to 7.7

Table 4 (b): Outcome According to CD34 Count

	Low CD34 (n=11)	High CD34 (n=10)	CI 95%
BM Cellularity	25% (5-40)	15% (5-30)	-9.3 to 23.6
Ferritin ng/mL	1051 (397-1686)	1373 (534-3959)	-1509 to 138.7
Conditioning n (%)			
FLU/CYC	10 (91)	10 (100)	
BU/CYC	1 (9)	0	
GVHD Prophylaxis			
CSA+MTX	8 (72)	4 (40)	
CSA+MP	3 (27)	7 (70)	
Engraftment day	labs	median (range)*	
Engraftment Day	18 (15-23)	20 (16-22)	
WBC x 10 ⁹ /L	5.2 (2-8.6)	3.8 (1.3-5.7)	-0.3 to 3
ANC x 10 ⁹ /L	1.5 (0.5-2.2)	2.1 (0.8-2.3)	0.0004 to 1.3
HB g/dl	9.5 (8.2-10)	9 (7.8-10)	-0.4 to 1.02
Platelet x 10 ⁹ /L	32 (15-111)	107 (42-156)	-99 to -26
HG % Mean	3	0.7	-5 to 0.5
Complications			
GVHD n (%)			
acute GVHD/0-1	2 (18)	2 (20)	
aGVHD/2-4	2 (18)	2 (20)	
De Novo GVHD	1 (9)		
2nd GVHD	2 (18)	1 (10)	
Infection	7 (64)	7 (70)	
Day Median (Range)*	(-3) to (-11)	(2) to (-13)	-4.2 to 1.4
Line 1 (TZP/ Gen/ Amph) n (%)	5 (45)	3 (30)	
Line 2 (Ipm/ Vori) n (%)	2 (18)	4 (40)	
Median Follow Up	39 mo.	65 mo.	-48.2 to 15

ANC, Absolute Neutrophil Count; BM, Bone Marrow; BSA, Body Surface Area; BU, Busulphan CI, Confidence Interval; CSA, Cyclosporine; CMV, Cytomegalovirus; CYC, Cyclophosphamide; FLU, Fludarabine; a/GVHD, acute/Graft Versus Host Disease; HB, Hemoglobin; M±SD, Mean ± Standard Deviation, mo., Months MP, Methylprednisolone; MTX, Methotrexate; WBC, White Blood Cells. *High CD34 ≥ 6.6 x 10⁶ cells/Kg, Low CD34 < 6.6 x 10⁶ cells/Kg. Range (minimum-Maximum). Student T Test.

Interestingly, studying two different GVHD prophylaxis used in our study; CSA+MTX (n=12) vs. CSA+MP (n=8) aiming to improve patients' outcome, patients who received CSA+MTX showed delayed engraftment (median day; 22, range; 16-27 vs. 17, 15-21; 95% CI; -5.7 to -0.6, $p=0.01$) and platelet count on engraftment (median; 99 x 10⁹ /L, range; 21-156 vs. 32, 15-103, 95% CI; -96 to -11.7, $p=.01$; Supplementary Table 5). Interestingly patients who got CSA + MTX

had frequently more patients with high HG%, above best cutoff values ($P=0.004$; Figure 4B) compared to those patients who got CSA+MP. Moreover, comparison of changes in pre- and post-transplantation peripheral blood counts shown in Figure 4C, indicated that platelet improvement was the only parameter in blood counts where there were significant changes in post-transplant values ($P=0.0001$) in patient got CSA+MTX regimen for GVHD prophylaxis

Supplementary Table 5 (a): Outcome According to GVHD Prophylaxis

	MTX+CSA(n=12)	MP+CSA(n=9)	CI 95%
Age M±SD	19±6.3	18±6.8	-7 to 5.1
Gender (male) n (%)	10 (83%)	7 (78%)	
BSA M±SD	1.6±0.5	1.5±0.2	-0.5 to 0.28
Screening n (%)			
A	7 (58%)	2 (22%)	
B	2 (17%)	2 (22%)	
O	3 (25%)	4 (44%)	
AB	0	1 (11%)	
Hepatitis B (HBsAg)	4 (33%)	2 (22%)	
CMV (IgG)	10 (83%)	9 (100%)	
CMV (IgM)	1 (8%)	0	
Toxoplasma (IgG)	5 (42%)	1 (11%)	
Pre-transplant Labs Median (Range)*			
WBC x 10 ⁹ /L	2.8 (1.2-4.3)	1.8 (0.7-4.1)	-1.8 to 0.2
ANC x 10 ⁹ /L	0.8 (0.3-1.2)	0.4 (0.1-1)	-0.6 to -0.01
HB g/dl	7.25 (3.8-14)	7.5 (5-9.3)	-3 to 1.7
Platelet x 10 ⁹ /L	8 (7-115)	10 (4-44)	-37 to 18
BM Cellularity	15 (0-40%)	25 (5-30)	-13.3 to 20.7
Ferritin ng/mL	1219 (397-3959)	1101 (675-1686)	-1296 to 513
CD34cells x 10 ⁶ cells/Kg	6 (3-15.8)	7 (3-7.5)	-4.2 to 2.5
Engraftment Labs Median (Range)*			
Engraftment Day	22 (16-27)	17 (15-21)	-5.7 to -0.6
WBC x 10 ⁹ /L	3.8 (1.3-8.6)	5 (3.6-6.5)	-0.9 to 2.6
ANC x 10 ⁹ /L	1.5 (0.5-3)	2 (1.4-2.6)	-0.28 to 1
HB g/dl	9.2 (8.3-10)	9.4 (7.8-10)	-0.9 to 0.5
Platelet x 10 ⁹ /L	99 (21-156)	32 (15-103)	-96 to -11.7
Complication n (%)			
GVHD			

SUPPLEMENTARY Table 5 (b): Outcome According to GVHD Prophylaxis

	MTX+CSA(n=12)	MP+CSA(n=9)	CI 95%
Acute GVHD/0-1	2 (17%)	2 (22%)	
aGVHD/2-4	2 (17%)	2 (22%)	
De novo GVHD	1 (8%)	0	
2nd GVHD	1 (8%)	2 (22%)	
Infection	9 (75%)	5(55%)	
Day Median (Range)	8 (-3-11)	5 (1-18)	
Line 1(TZP/ Gen/ Amph) n (%)	4 (33%)	4 (44%)	
Line 2(IMP / Vori) n (%)	5 (42%)	1 (11%)	
Median Follow Up	77mo.	34 mo.	-65.1 to 7.1

AMP, Amphotericin, ANC, Absolute Neutrophil Count, BM, Bone Marrow, BSA, Body Surface Area, BU, Busulfan, CI, Confidence Interval, CMV, Cytomegalovirus, CYC, Cyclophosphamide, FLU, Fludarabine, Gen, Gentamycin, A/GVHD; Acute/Graft Versus Host Disease, HB, Hemoglobulin, IMP, Imipenem, MP, Methylprednisolone, MTX, Methotrexate, TZP, Tazobactam, Vori, Voriconazole, WBC, White Blood Cells.

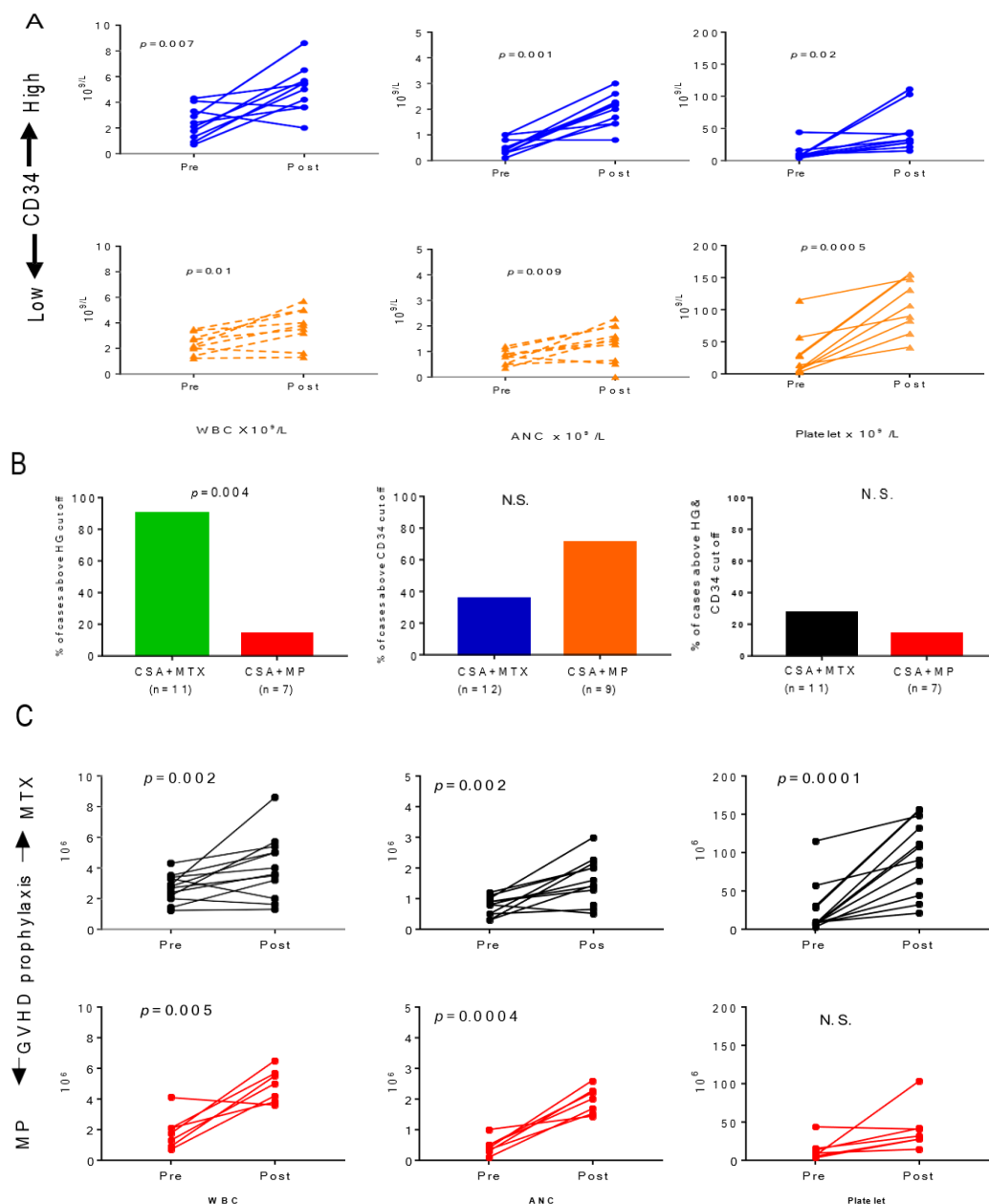


FIGURE 4: A) Comparison Between Pre-and Post-Transplant Count in Patients with High and Low CD34 Count. Upper Panel; Mean +SD of Differences Between Pre and Post Transplantation Counts in High CD34 Count; WBC, ANC, HB And Platelet. Lower Panel; Mean +SD Of Differences Between Pre and Post Transplantation Counts in Low CD34 Count. WBC, ANC, HB And Platelet. Student T Test with Significance $P < 0.05$. B) Relation Between Biological Indicators and GVHD Prophylaxis Regimens Used in Our Cohort. Comparing HG% And CD34 Count in Patient Received CSA+MTX And CSA+MP, Fisher Exact Test with Significance $P < 0.05$. C) Upper Panel; Mean +SD Of Differences Between Pre and Post Transplantation Counts in Patients Received CSA+MTX; WBC, ANC, HB And Platelet. Lower Panel; Mean +SD Of Differences Between Pre and Post Transplantation Counts in Patients Received CSA+MTP. WBC, ANC, HB And Platelet. ANC; Absolute Neutrophil Count, HB; Hemoglobin, HG; Hematogones, WBC. White Blood Cells, Student T Test with Significance $P < 0.05$

Impact of Prognostic Indicators on Overall Survival

The Kaplan-Meier probability of OS in this study showed significantly patients with high HG (n=11) has better OS ($p=0.005$; Figure 5A) compared to patients with low HG (n=7), neither ferritin

nor CD 34⁺ count impact the OS ($p=0.9$ and 0.1 respectively; Figure 5B, C) of SAA whom were treated with allo-HSCT. Patients who received CSA+MTX (n=12) had better OS compared patients who received CSA+MP (n=9, $p=.04$, Figure 5D).

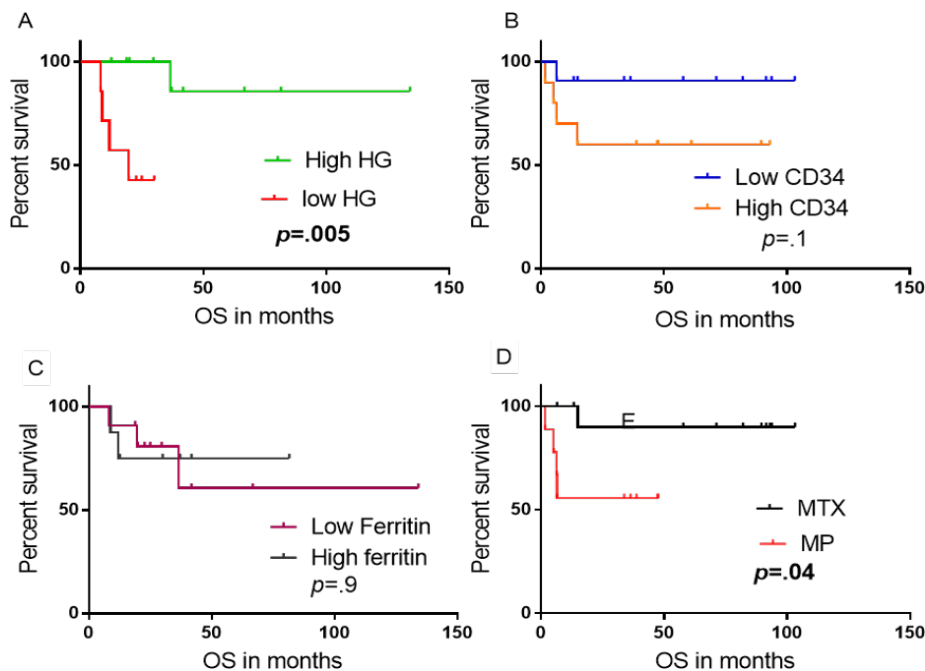


Figure 5: Kaplan Meyer Curves Show Difference Overall Survival A) Showing OS of the Patient Cohort Compares OS of Patients with High vs. Low HGs, B) Compares OS of Patients with High Ferritin Level vs. Low Ferritin Level, C) Compares OS of Patients with High CD34 Count vs. Low CD34. D) OS Compares Patients with High vs Low Ferritin Levels. MTX, Methotrexate, MP, Methylprednisolone, OS, Overall Survival. Log Rank Test, Significant $P<0.05$

Discussion

In this study, we have evaluated a flow cytometer HG 4-color assay at engraftment using CD10, CD19, CD20, CD22, CD34, and CD38 obtained on bone marrow aspirate and The prism function of the software was used to estimate the percentage of cell populations expressing different antigen combinations as majority of literature suggested [28] in comparison to infused CD34⁺ cell dose as well as serum ferritin levels as prognostic biomarkers in SAA to predict outcome of allogeneic HSCT using MSD.

Moreover, GVHD prophylaxis regimen evaluated in our cohort patients to examine its relationship with selected prognostic biomarkers used in this study.

Studying the outcome of allogeneic HSCT was important knowing that the overall incidence of AA was 2.34 cases per million per year with about 2/3 diagnosed as SAA, OS was 73% in 3 months, 57% in 5 years [2]. Although HSCT improved OS over IST [4, 45, 46]. HGs had an emerging role as a prognostic indicator in allogeneic HSCT that was not studied enough in SAA and never studied in eastern countries [24, 27, 28].

Limitations in our study were the small sample size as we concentrated only on patients diagnosed with SAA and received MSD allogeneic HSCT as well as difficulty to consent patients for bone marrow sampling post-transplantation and concise follow-up (median 47months). Most important would be a future longitudinal follow up study of HGs% overtime as well as other indicators improve HSCT outcome [47], even with using umbilical cord, and / or unrelated matched donors.

Infusion of high CD34⁺ especially peripheral blood HSCT in SAA helps rapid engraftment which leads to less treatment-related mortality although infusion of high number of CD34⁺ cells was shown to be associated with increased risk of chronic GVHD, a highly morbid sequel of BMT, hence optimization is required [18, 20]. In addition to ferritin level with different cut-off levels which act as independent risk factor of non-relapse mortality and overall survival post HSCT and on HSCT outcome [13, 33, 48].

Infused CD34⁺ count in allogeneic HSCT in SAA patients could be a reasonable predictor of engraftment [20, 34] which was demonstrated in our study using cut off $6.8 \times 10^6/L$ with 62% sensitivity and 80% specificity. Interestingly CD34⁺ count lower relapse rate and disease-free survival but on the contrary didn't affect the OS [34]. Ferritin as prognostic factors that we studied in our patients with cut-off 1337 ng/dl, the majority of literature started to consider the adverse effect of ferritin in the level above 1000 ng/dl [33, 34]. Ferritin didn't show the impact on overall survival although in some studies ferritin impact outcome with higher cut off, interestingly there is a higher risk of mold infection with higher levels of ferritin [48, 49].

In this study; a defined a cut-off level of HG in patients diagnosed with SAA, treated with allogeneic HSCT from MSD (0.37% of total MNCs) with 77% sensitivity and 80% specificity. HG cut off levels used to range from 0.25 % to 5% in previous studies that discussed myeloid neoplasms treated with HSCT from different stem cells source [24, 27, 28, 42].

Median time of HG appearance was day 19 (ranges from 15 to 23) which was consistent with the previous studies [24]. Patients with high HG had better WBC, ANC and platelet count on engraftment in addition

to better OS, which was the point of controversy. As in previous studies of HG post HSCT in AML patients; HG was a positive prognostic indicator [27, 28, 43]. While in studies using single cord blood unit as a source of stem cells, HG didn't show any advantage [42]. B cell neoplasm studies showed an adverse effect of high HG after autologous HSCT [50]. Noticeably donor age was significantly inverse proportion with HG count similarly to our cohort although didn't come out significant.

Moreover, many studies showed that high HG is associated with less acute, chronic GVHD and post-transplantation infections [24, 27, 28] which was not shown in our data; due to sample size and as in Hiroto Ishii et.al; 2017 which showed that higher hematogones levels are not correlated with better recovery of B lymphocytes post-transplantation.

In this study one of the interesting clinical observations was the difference in the outcome of patients who got two GVHD prophylaxis regimen CSA+MTX and CSA +MP; patients received MP had significant earlier engraftment and worse OS with no difference in the incidence of GVHD consistent with other studies [35, 51]. On further

study, MTX showed increased frequency of patients with high HGs levels compared to MP based regimen for GVHD prophylaxis.

Uniquely previous literature that handle aplastic anemia and HG highlighted the diagnostic use of HG to different AA and MDS with low HG count but not as a prognostic biomarker for SAA patients treated with allogeneic HSCT [52].

In conclusion; total HGs showed the best sensitivity and specificity as a predictor biomarker for HSCT outcome in SAA patients among other prognostic indicators that we studied. Moreover, HG% significantly impacted on SAA survival following MSD-all-HSCT. Using MTX in GVHD prophylaxis may increase total HGs% and improve survival in SAA patients. HGs should be studied on a wider scale and for longer follow-up duration.

Acknowledgment

This research was supported by financial support of Faculty of Medicine, Assiut University, South Egypt cancer institute in collaboration with Nasser Institute for research and treatment in Egypt and Ministry of Higher Education, Joint supervision department.

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