High expression of the stem cell marker GPR56 at diagnosis identifies acute myeloid leukemia patients at higher relapse risk after allogeneic stem cell transplantation with the CD34$^+$/CD38$^-$ population

In patients with acute myeloid leukemia (AML), leukemia-initiating cells (LIC) are believed to be responsible for disease initiation, maintenance, and relapse. While the highest frequency of LIC exists in the CD34$^+$/CD38$^-$ bone marrow (BM) compartment,[1] over the past years it has become evident that a subset of the CD34$^-$ and/or CD38$^+$ population may also harbor AML stem cell potential.[2,3] LIC phenotypes also show inter-individual heterogeneity dependent on genetic subtypes,[4] and new potential LIC markers might better define this population. The G protein-coupled adhesion molecule GPR56 regulates survival, migration, and adhesion in various cell types.[5] GPR56 was shown to be upregulated in healthy hematopoietic stem cells as well as LIC, especially when residing in a quiescent state, and to be downregulated during hematopoietic maturation.[6] In AML, GPR56 may be important for the LIC - stem cell niche interaction.[7] With the ability to serially transplant leukemia in NOD/SCID mice, high GPR56 expressing AML cells were functionally validated as LIC.[8,9] This ability was also found in LIC with low or absent CD34 expression, supporting GPR56 as a marker able to identify LIC independent of the CD34 expression status.[8]

High *GPR56* expression has also been linked to a dismal prognosis in AML patients consolidated with chemotherapy.[8-11]

Recently, we demonstrated that a high CD34$^+$/CD38$^-$ BM cell burden (≥6%) at diagnosis is associated with shorter survival in AML patients receiving allogeneic hematopoietic stem cell transplantation (HSCT).[12] While the outcome was dismal in most patients with a high CD34$^+$/CD38$^-$ cell burden, outcome remained heterogeneous in the large patient group (85%) with a low CD34$^+$/CD38$^-$ cell burden at diagnosis, ranging from relapse 1 month after HSCT to 10 years disease-free follow up.

Here, we retrospectively evaluated the prognostic impact of a differential *GPR56* expression additionally to the CD34$^+$/CD38$^-$ cell burden in 213 AML patients for whom BM aspirate material at diagnosis was available. All patients were treated with cytarabine-based chemotherapy and consolidated with non-myeloablative (NMA; 72%) or myeloablative (MAC; 28%) HSCT in complete remission (CR; 87%) or CR with incomplete peripheral recovery (CRi; 13%). For further details see the *Online Supplementary Materials and Methods*. Patients' characteristics are shown in Table 1 and in the *Online Supplementary Tables S1-2*. Median follow up after HSCT for patients alive was 5.2 years.

At diagnosis, *GPR56* expression and the expression of an internal control gene (*ABL1*) were determined using quantitative real time PCR in all patients and a median cut-off of the normalized gene expression was used to define high and low *GPR56* expressers. In a patient subset GPR56 expression assessed by flow cytometry correlated well with *GPR56* expression (R^2=0.80; *Online Supplementary Figure S1*). Flow cytometry determined the CD34$^+$/CD38$^-$ cell burden in patients with data available (n=180) and the previously evaluated 6% cut[12] defined patients with a high or low CD34$^+$/CD38$^-$ cell burden. Karyotypes, the mutation status of 52 recurrently mutated genes in AML, and expression levels of *BAALC, MN1*, and *EVI1* were evaluated at diagnosis in patients with available material (see the *Online Supplementary Material and Methods*).

Previously, a high *GPR56* expression has been linked to AML with adverse cytogenetic or molecular risk, especially to anomalies of chromosomes 5 or 7, high *EVI1* expression or the presence of *RUNX1* mutations.[7,8] EVI1[7] and RUNX1[6] were also described to directly target *GPR56*. In line with these findings, we observed a higher incidence of deletion (del) 7/7q (P=0.003), del5/5q (P=0.005), complex (P=0.04) and monosomal (P=0.02) karyotypes, higher frequency of *RUNX1* mutations (P=0.05) and more *EVI1* positive cases (P=0.02) in high *GPR56* expressing patients. In contrast, a low *GPR56* expression was previously described in core-binding factor (CBF) AML, AML with *NPM1* mutations without the presence of a *FLT3*-ITD and AML with monocytic/monoblastic morphology.[8,9] Consistently, we observed a lower incidence of French American British M5 (P=0.003) cases, CBF AML (P=0.003) as well as *NPM1* mutations (P=0.001) and *NPM1* mutations without *FLT3*-ITD (P<0.001) in patients with high *GPR56* expression. We could not confirm the previously described high *GPR56* expression in patients with mutated *NPM1* and *FLT3*-ITD (*GPR56* as continuous variable, P=0.37), but only 18 *NPM1* mutated patients in our set harbored a *FLT3*-ITD. Additionally, we observed an asso-

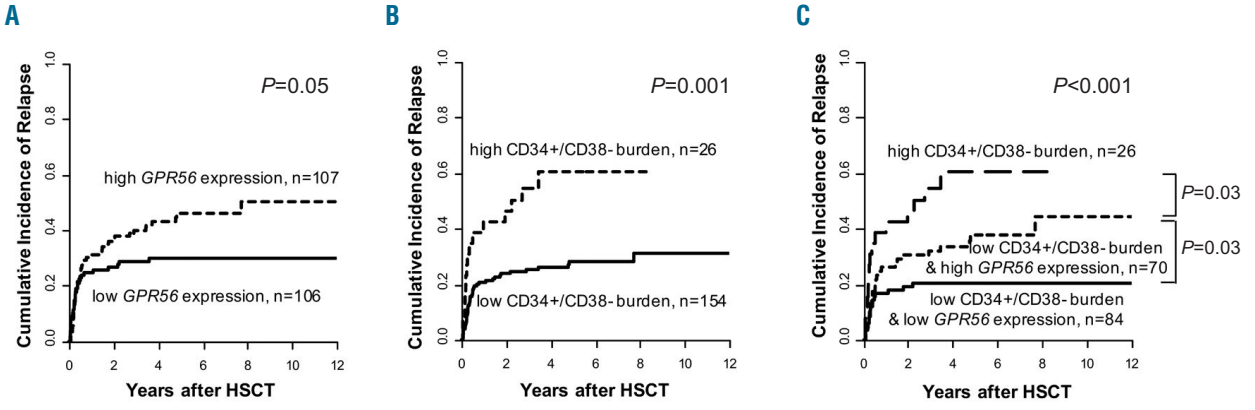

Figure 1 Cumulative incidence of relapse according to leukemia initiating cell burden. (A) according to the *GPR56* expression at diagnosis, high *vs.* low, median cut; (B) according to the CD34$^+$/CD38$^-$ cell burden at diagnosis, high *vs.* low, 6% cut; (C) according to the CD34$^+$/CD38$^-$ cell burden and the *GPR56* expression.

Table 1. Clinical and genetic characteristics of acute myeloid leukemia patients treated with hematopoietic stem cell transplantation according to the *GPR56* expression at diagnosis, median cut, n=213.

Characteristic	All patients (n=213)	Low *GPR56* expression (n=106)	High *GPR56* expression (n=107)	*P*
Clinical Characteristics				
Age at diagnosis, years				
Median	59.7	58.4	60.7	0.14
Range	14.5-75.8	14.5-74.8	18.5-75.8	
Sex, n (%)				
Male	104	52 (49)	52 (49)	1
Female	109	54 (51)	55 (51)	
Disease origin, n (%)				
De novo	149	82 (77)	67 (63)	0.03
Secondary	64	24 (23)	40 (37)	
Hemoglobin, g/dL				
Median	8.9	9.2	8.6	0.14
Range	4.3-15.7	4.5-15.7	4.3-14.9	
Platelet count, x 10^9/L				
Median	63	58	65	0.40
Range	1-327	1-327	3-305	
WBC count, x 10^9/L				
Median	7.5	14.2	4.5	0.002
Range	0.7-385	0.9-385	0.7-295	
Percentage of blood blasts, %				
Median	26	32	21	0.21
Range	0-98	0-97	0-98	
Percentage of BM blasts, %				
Median	58	60	57	0.90
Range	0-95	10-95	0-95	
BM CD34+/CD38- burden, %				
Median	0.5	0.2	1	<0.001
Range	0-89	0-75	0-89	
Genetic Characteristics				
Karyotype, n (%)				
Abnormal	115	50 (51)	65 (63)	0.09
Normal	86	48 (49)	38 (37)	
Monosomal karyotype, n (%)				
Absent	174	92 (94)	82 (82)	0.02
Present	24	6 (6)	18 (18)	
Complex karyotype, n (%)				
Absent	169	89 (91)	80 (80)	0.04
Present	29	9 (9)	20 (20)	
del5/del(5q), n (%)				
Absent	189	100 (97)	89 (86)	0.005
Present	18	3 (3)	15 (14)	
del7/del(7q), n (%)				
Absent	177	96 (93)	81 (78)	0.003
Present	30	7 (7)	23 (22)	
CBF AML, n (%)				
Absent	181	83 (85)	98 (97)	0.003
Present	18	15 (15)	3 (3)	
Trisomy 8, n (%)				
Absent	183	93 (90)	90 (87)	0.52

Present	24	10 (10)	14 (14)	
ELN 2017 Genetic Group, n (%)				
Favorable	55	43 (60)	12 (15)	<0.001
Intermediate	23	6 (8)	17 (23)	
Adverse	65	23 (32)	42 (58)	
NPM1, n (%)				
Wild-type	164	71 (67)	93 (87)	0.001
Mutated	49	35 (33)	14 (13)	
FLT3-ITD, n (%)				
Absent	174	89 (84)	85 (79)	0.48
Present	39	17 (16)	22 (21)	
NPM1 mut/no *FLT3*-ITD, n (%)				
Absent	182	82 (77)	100 (93)	<0.001
Present	31	24 (23)	7 (7)	
CEBPA, n (%)				
Wild-type	172	86 (91)	86 (92)	0.50
Single mutated	11	5 (5)	6 (6)	
Double mutated	5	4 (4)	1 (1)	
DNMT3A, n (%)				
Wild-type	64	30 (73)	34 (81)	0.44
Mutated	17	11 (27)	8 (19)	
FLT3-TKD, n (%)				
Wild-type	178	93 (89)	85 (86)	0.52
Mutated	25	11 (11)	14 (14)	
IDH1, n (%)				
Wild-type	71	37 (90)	34 (81)	0.35
Mutated	12	4 (10)	8 (19)	
IDH2, n (%)				
Wild-type	70	34 (81)	36 (86)	0.77
Mutated	13	7 (19)	6 (14)	
JAK2, n (%)				
Wild-type	76	41 (100)	35 (83)	0.01
Mutated	7	0 (0)	7 (17)	
RUNX1, n (%)				
Wild-type	72	39 (95)	33 (79)	0.05
Mutated	11	2 (5)	9 (21)	
TP53, n (%)				
Wild-type	75	38 (90)	37 (90)	1
Mutated	8	4 (10)	4 (10)	
ASXL1, n (%)				
Wild-type	69	32 (79)	37 (88)	0.25
Mutated	14	9 (21)	5 (12)	
EVI1 expression status, n (%)				
Negative	118	65 (89)	53 (74)	0.02
Positive	27	8 (11)	19 (26)	
MN1/ABL1 expression				
Median	0.12	0.02	0.26	<0.001
Range	0.00-59.67	0.00-1.63	0.00-59.67	
BAALC/ABL1 expression				
Median	0.08	0.01	0.20	<0.001
Range	0.00-56.3	0.00-2.41	0.00-56.3	

ABL1: Abelson murine leukemia viral oncogene homolog 1 gene; AML: acute myeloid leukemia; *BAALC*: brain and acute leukemia cytogenetic gene; *BCOR*: BCL6 corepressor gene; BM: bone marrow; CBF: core-binding factor; *CEBPA*: CCAAT/enhancer-binding protein alpha gene; del: deletion; *DNMT3A*: DNA-methyltransferase 3A gene; ELN: European LeukemiaNet; EVI1: ecotropic viral integration site gene; *FLT3*-ITD: internal tandem duplication of the *FLT3* gene; *FLT3*-TKD: tyrosine kinase domain of the *FLT3* gene; HSCT: hematopoietic stem cell transplantation; *IDH1*: isocitrat dehydrogenase 1 gene; *IDH2*: isocitrat dehydrogenase 2 gene; *JAK2*: janus kinase 2 gene; *MN1*: meningioma-1 gene; *NPM1*: nucleophosmin-1 gene; *RUNX1*: Runt-related transcription factor 1 gene; *TP53*: tumor protein 53; WBC: white blood cell.

Table 2. Multivariable outcome analysis of 180 acute myeloid leukemia patients treated with hematopoietic stem cell transplantation according to the *GPR56* expression and the CD34$^+$/CD38$^-$ cell burden at diagnosis.

Variable	Cumulative incidence of Relapse			Leukemia Free Survival		
	HR*	95% CI	*P*	OR**	95% CI	*P*
Leukemia initiating cell population (low CD34$^+$/CD38$^-$ & low *GPR56 vs.* low CD34$^+$/CD38$^-$ & high *GPR56 vs.* high CD34$^+$/CD38$^-$)	1.77	1.12-2.80	0.02	0.67	0.51-0.89	0.007
EVI1 expression at diagnosis (negative *vs.* positive)	2.54	1.27-5.08	0.008	–	–	–
Age at HSCT, years	–	–	–	0.97	0.95-0.99	0.008
Platelet count at diagnosis, Gpt/l	–	–	–	1.00	1.00-1.01	0.03

CI: confidence interval; HSCT: hematopoietic stem cell transplantation; HR: hazard ratio; OR**: odds ratio, <1 (>1) indicate lower (higher) risk for an event for the first category listed for the dichotomous variables and for the higher values of the continuous variables. Variables considered in the models were those significant at =0.20 in univariable analyses. Variables considered for CIR were: disease origin (secondary *vs. de novo*), ELN 2017 genetic group, platelet count at diagnosis, leukemia initiating cell population at diagnosis (low CD34$^+$/CD38$^-$ & low *GPR56 vs.* low CD34$^+$/CD38 & high *GPR56 vs.* high CD34$^+$/CD38$^-$), *EVI1* expression at diagnosis (negative *vs.* positive), *BAALC* expression at diagnosis (negative *vs.* positive), *MN1* expression at diagnosis (negative *vs.* positive). Variables considered for LFS were: disease origin (secondary *vs. de novo*), ELN 2017 genetic group, platelet count at diagnosis, bone marrow blast count at diagnosis, leukemia initiating cell population at diagnosis (low CD34$^+$/CD38$^-$ & low *GPR56 vs.* low CD34$^+$/CD38$^-$ & high GPR56 *vs.* high CD34$^+$/CD38$^-$), *EVI1* expression at diagnosis (negative *vs.* positive) and age at HSCT.

ciation of high *GPR56* expression with lower white blood count (*P*=0.002), secondary AML (*P*=0.03), and an adverse ELN 2017[13] genetic risk (*P*<0.001), more *JAK2* mutated AML (*P*=0.01), and higher *BAALC* and *MN1* expression levels (*P*<0.001 and *P*<0.001, respectively) at diagnosis. High *GPR56* expressing patients also showed a higher CD34$^+$/CD38$^-$ cell burden at diagnosis (*P*<0.001) and a distinct immunophenotype (see the *Online Supplementary Materials and Methods*). Similar to patients consolidated with chemotherapy,[8-10] a high *GPR56* expression associated with a higher cumulative incidence of relapse (CIR; *P*=0.05, Figure 1A) in patients receiving HSCT. As expected, a high CD34$^+$/CD38$^-$ cell burden also associated with a higher CIR in the here presented patient population (*P*=0.001, Figure 1B). As described above, outcome in the low CD34$^+$/CD38$^-$ cell burden group remained heterogeneous, but no further prognostic impact was observed introducing an additional 1% or 2% cut for the CD34$^+$/CD38$^-$ cell burden (*Online Supplementary Figure S2*). We hypothesized that this may be because of a LIC population not defined by the CD34$^+$/CD38$^-$ phenotype and that *GPR56* – described to define LIC independently of the CD34$^+$/CD38$^-$ phenotype[8] - might identify individuals at higher relapse risk in patients with a low CD34$^+$/CD38$^-$ cell burden (n=154). Interestingly, in the low CD34$^+$/CD38$^-$ cell burden group, a high *GPR56* expression identified AML patients with a higher CIR than those with low *GPR56* expression (*P*=0.03), but a lower CIR than those with a high CD34$^+$/CD38$^-$ cell burden (*P*=0.03, Figure 1C). Thus, the *GPR56* expression may add further prognostic information to the established CD34$^+$/CD38$^-$ LIC phenotype. In multivariable analysis, a higher LIC burden defined by the CD34$^+$/CD38$^-$ cell burden and *GPR56* expression at diagnosis significantly associated with a higher CIR after adjustment for *EVI1* expression status and shorter leukemia free survival (LFS) after adjustment for platelet count at diagnosis and age at HSCT (Table 2). The prognostic significance of the CD34$^+$/CD38$^-$ cell burden and the *GPR56* expression levels were also confirmed in an independent patient cohort receiving HSCT, as well as a second validation cohort consolidated with chemotherapy (see *Online Supplementary Materials and Methods* and *Online Supplementary Figure S3-4*), and a third validation cohort using mRNA data within the TCGA dataset (*Online Supplementary Figure S5*).

In previous studies, the dismal prognostic impact of a high *GPR56* expression was shown in patients consolidated with chemotherapy[8-10] and as part of a 17-gene stemness score for outcome also in patients consolidated with HSCT.[14] To our knowledge, we are the first to analyze *GPR56* expression levels as a single marker and in context of the CD34$^+$/CD38$^-$ cell burden at diagnosis and its impact on the outcome after HSCT, a procedure that relies on immunological graft-*versus*-leukemia (GvL) effects. In line with previously published data, we observed associations with adverse-risk and immature genetic and immunophenotypic markers as well as a higher CIR in patients with high *GPR56* expression. Despite a correlation between the CD34$^+$/CD38$^-$ cell burden and *GPR56* expression levels, a high *GPR56* expression identified individuals with a higher CIR within the low CD34$^+$/CD38$^-$ cell burden cohort, subsequently adding valuable prognostic information to the CD34$^+$/CD38$^-$ phenotype.

Our findings underline the reduced immunogenicity of LIC[15] and indicate that the GvL effects after HSCT may not be sufficient to prevent high *GPR56* expressing patients from relapse after HSCT. Despite the lack of difference in non-relapse mortality (*Online Supplementary Figure S6A*) and a separation of the LFS curves, we observed no significant impact of high *GPR56* expression on LFS (*P*=0.14, *Online Supplementary Figure S6B*) in univariable analysis. The GvL effects after HSCT may contribute to an improved success of relapse therapy consisting of cytotoxic treatment, reduction of immunosuppressive drugs and/or donor lymphocyte infusion. Therefore, GvL effects might not fully overcome the inferior prognosis associated with a high CD34$^+$/CD38$^-$ cell burden,[12] but may have this potential in patients with low CD34$^+$/CD38$^-$ cell burden but high *GPR56* expression. Prospective clinical studies are needed to further address this point before implementation into clinical practice. Finally, beyond their role as a prognostic factor in AML, targeted therapies against the LIC population, including GPR56, might improve patients' outcome.

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