SUPPLEMENTARY APPENDIX

Immunophenotypic analysis of erythroid dysplasia in myelodysplastic syndromes. A report from the IMDSFlow working group

Theresia M. Westers,[1] Eline M.P. Cremers,[1] Uta Oelschlaegel,[2] Ulrika Johansson,[3] Peter Bettelheim,[4] Sergio Matarraz,[5] Alberto Orfao,[5] Bijan Moshaver,[6] Lisa Eidenschink Brodersen,[7] Michael R. Loken,[7] Denise A. Wells,[7] Dolores Subirá,[8] Matthew Cullen,[9] Jeroen G. te Marvelde,[10] Vincent H.J. van der Velden,[10] Frank W.M.B. Preijers,[11] Sung-Chao Chu,[12] Jean Feuillard,[13] Estelle Guérin,[13] Katherina Psarra,[14] Anna Porwit,[15,16] Leonie Saft,[16] Robin Ireland,[17] Timothy Milne,[17] Marie C. Béné,[18] Birgit I. Witte,[19] Matteo G. Della Porta,[20] Wolfgang Kern[21] and Arjan A. van de Loosdrecht,[1] on behalf of the IMDSFlow Working Group

[1]Department of Hematology, VU University Medical Center, Cancer Center Amsterdam, The Netherlands; [2]Department of Internal Medicine, Universitätsklinikum "Carl-Gustav-Carus", Dresden, Germany; [3]Department of Haematology, University Hospitals NHS Foundation Trust, Bristol, UK; [4]First Medical Department, Elisabethinen Hospital, Linz, Austria; [5]Servicio Central de Citometría (NUCLEUS) and Department of Medicine, Centro de Investigación del Cáncer, Instituto de Biologia Celular y Molecular del Cáncer, (CSIC/USAL and IBSAL), Universidad de Salamanca, Spain; [6]Isala Clinics, Zwolle, The Netherlands; [7]HematoLogics, Inc., Seattle, WA, USA; [8]Department of Hematology, Hospital Universitario de Guadalajara, Spain; [9]HMDS, St. James's University Hospital, Leeds, UK; [10]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, The Netherlands; [11]Department of Laboratory Medicine – Laboratory for Hematology, Radboud University Medical Center, Nijmegen, The Netherlands; [12]Department of Hematology and Oncology, Buddhist Tzu Chi General Hospital, Tzu Chi University, Hualien, Taiwan; [13]Laboratoire d'Hématologie, CHU Dupuytren, Limoges, France; [14]Department of Immunology-Histocompatibility, Evangelismos Hospital, Athens, Greece; [15]Department of Pathobiology and Laboratory Medicine, University of Toronto, University Health Network, Toronto General Hospital, ON, Canada; [16]Department of Pathology, Karolinska University Hospital, Stockholm, Sweden; [17]King's College Hospital, London, UK; [18]Laboratoire d'Hématologie, CHU de Nantes, France; [19]Department of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands; [20]Department of Hematology and Oncology, Fondazione IRCCS Policlinico San Matteo, and University of Pavia, Italy and [21]MLL Munich Leukemia Laboratory, Germany

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Correspondence: tm.westers@VUmc.nl

Supplementary information

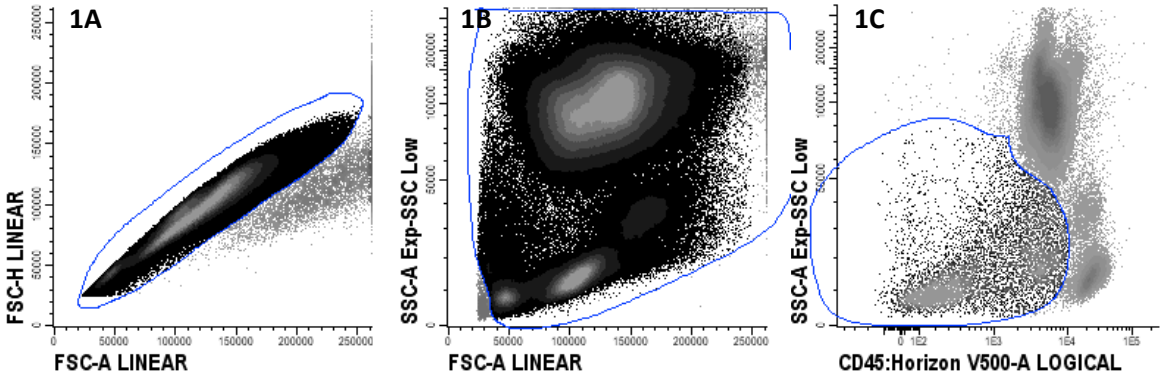
TM Westers et al.: Immunophenotypic analysis of erythroid dysplasia in myelodysplastic syndromes. A report from the IMDSFlow working group.

Proposed gating strategy and recommended parameters for analysis of the erythroid lineage

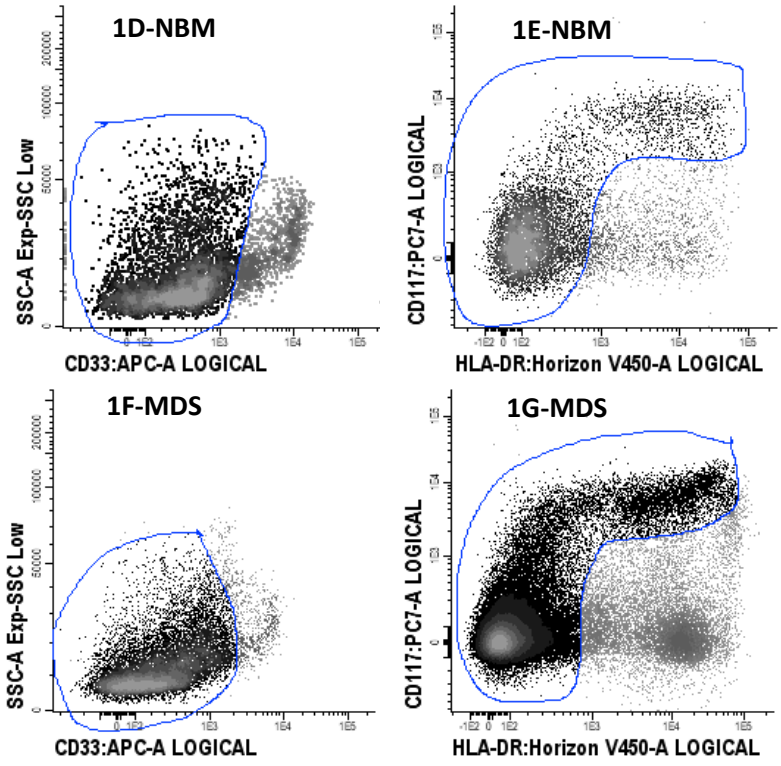
All data were generated on a FACS CANTO-II flow cytometer (BD Biosciences, La Jolla, CA); plots have been generated using Infinicyt Software, Cytognos, Salamanca, Spain)

1. Percentage of nucleated erythroid cells of total nucleated cells

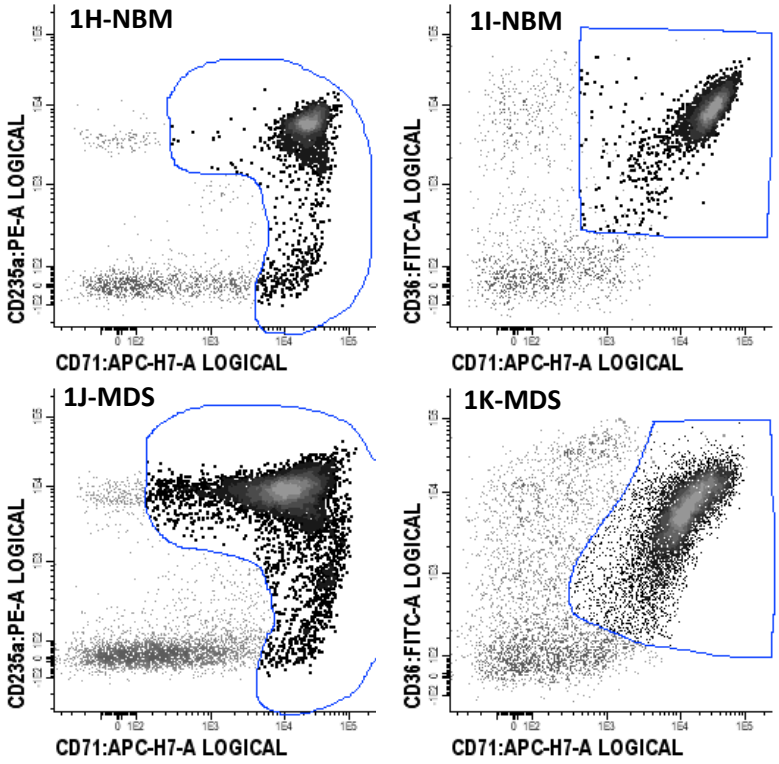
- Select singlets in FSC-A vs. FSC-H plot (when possible); figure 1A.
- Exclude debris in FSC-SSC plot (when available a nuclear dye can be helpful); figure 1B.
- Select CD45$^{negative\text{-}to\text{-}dim}$ with SSC$^{low\text{-}to\text{-}int}$ in CD45 vs. SSC plot; figure 1C.



- CD45dim myeloid and B cell precursors are present in the current selected population, therefore … Exclude remaining myeloid cells by e.g. the selection of CD33 negative cells or CD13 negative cells (not shown). An example is shown for a normal bone marrow (NBM) sample and a MDS sample (figures 1D and F). B cell progenitors and other contaminating non erythroid cells may be excluded by their CD117^{-}HLA-DR^{+} phenotype (figures 1E and G).



- Next, exclude remaining mature erythrocytes (CD235a^{+}CD71^{-} or CD36^{-}CD71^{-}) and platelets (CD36+CD71-); figures 1H-K



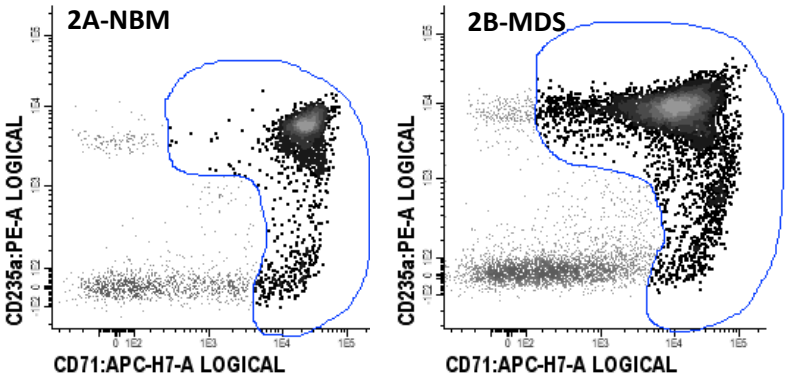
- Express the remaining immature and maturing erythroid cells as percentage of total nucleated cells ((doublets and) debris excluded) : **% erythroid of nucleated cells**

  Note!

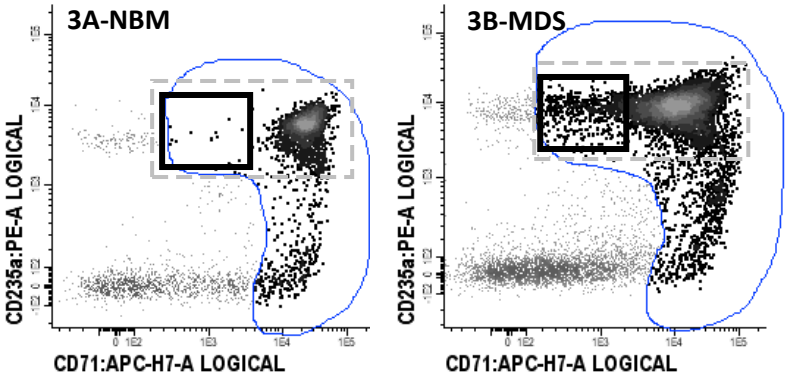
  a. You can also select CD235a^{+}CD71^{+} and CD235^{+}CD71^{dim} according to Matarraz et al., Cytometry Part B: Clinical Cytometry 2010, 78B, pages 154–168: CD117^{-} nucleated erythroid cells (examples: solid line rectangular boxes in figures 4A and 4B); and enumerate CD117+ precursors separately. Both added together represent **% erythroid of nucleated cells**

  b. When CD235a is not available for analysis, enumerate erythroid cells by CD71 (and CD105) expression in the CD45$^{negative\text{-}to\text{-}dim}$ subpopulation (note to exclude myeloid precursors

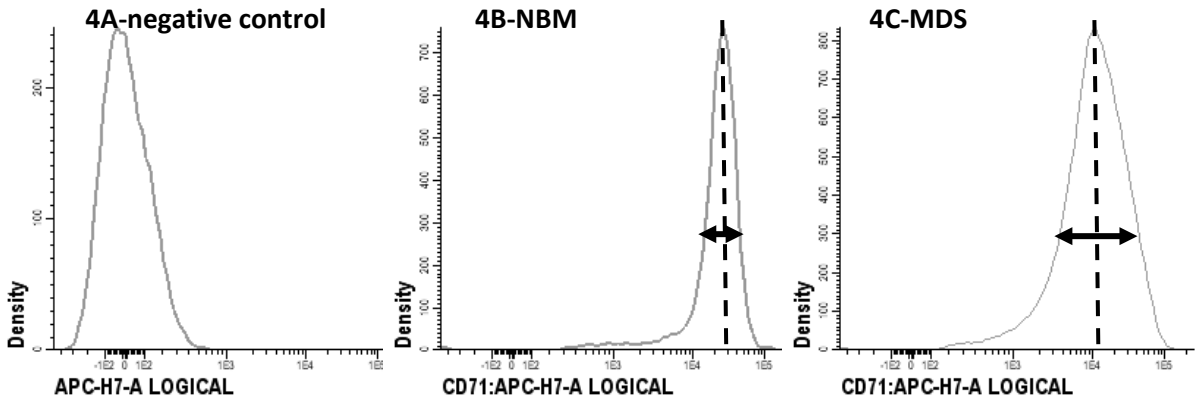
2. Interpret the CD71 vs. CD235a erythroid differentiation pattern as normal or aberrant according to your own center's reference profiles; Figures 2A and 2B
This can be performed by "eye balling", by automated reference plots, by occurrence of a $CD71^{dim}$ population, by increase in CV of CD71 expression or altered mean fluorescence intensity, by altered percentage of precursors ($CD71^{+}CD235a^{-}$). Some of these features are described below.



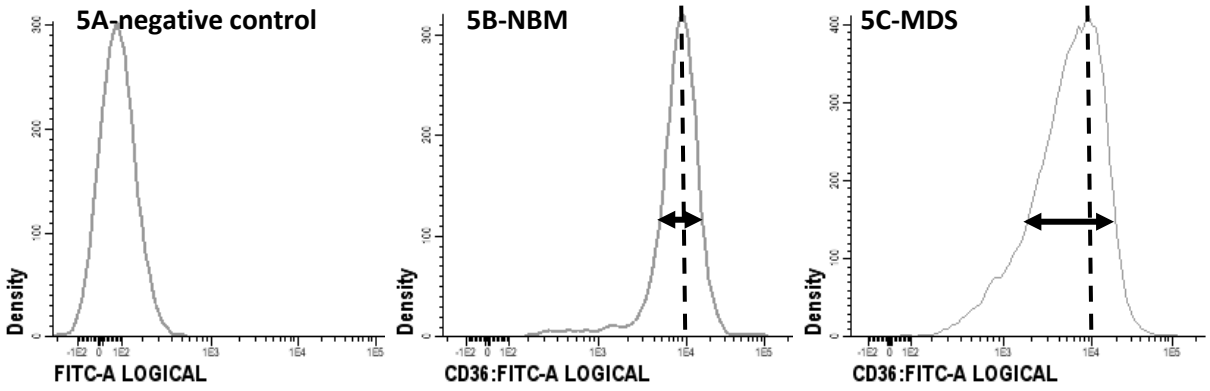
3. $CD71^{dim}$ fraction as percentage of the $CD235a^{+}$ $CD71^{+}$ erythroid cells. The $CD71^{dim}$ fraction is mostly absent in NBM and may be present in MDS (boxes in figures 3A and B). The $CD71^{dim}$ fraction may also be evaluated in the CD71 vs. CD36 plot (Figures 1I and 1K)



4. CD71 expression. CD71 expression level can be analyzed as fluorescence intensity or coefficient of variation (CV). The CV represents a homogenous or aberrantly heterogeneous expression profile (arrows). With regard to expression level, preferably use geo mean of fluorescence intensity as compared to unstained cells (or an irrelevant antibody as back ground/auto fluorescence; figure 4A); use median if geo mean is not available. Examples in figures 4B and 4C. Erythroid cells in MDS-example in figure 4C display lower expression of CD71 and increased CV (more heterogeneous) as compared to NBM in figure 4B.

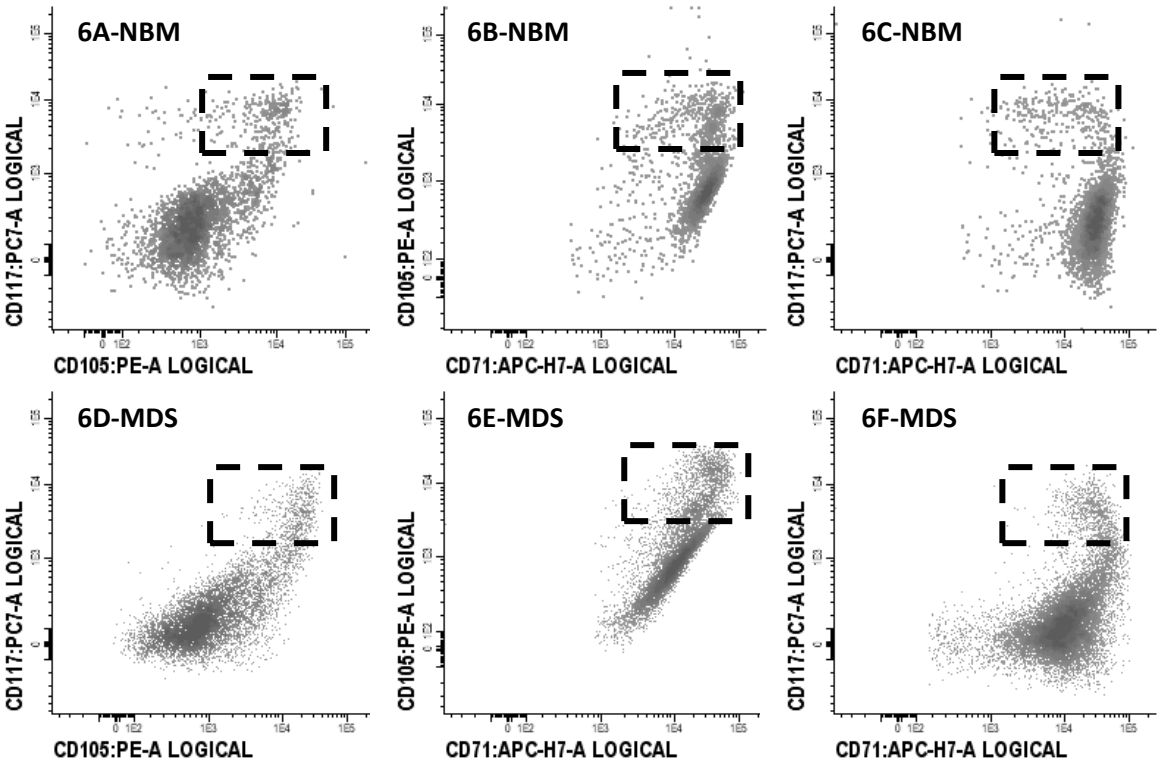


5. CD36 expression. CD36 expression level can be analyzed as fluorescence intensity or coefficient of variation (CV). With regard to expression level, preferably use geo mean of fluorescence intensity as compared to unstained cells (or an irrelevant antibody as back ground/auto fluorescence; figure 5A); use median if geo mean is not available. Examples in figures 6B and 6C. Erythroid cells in MDS-example in figure 5C display increased CV (more heterogeneous) as compared to NBM in figure 5B.

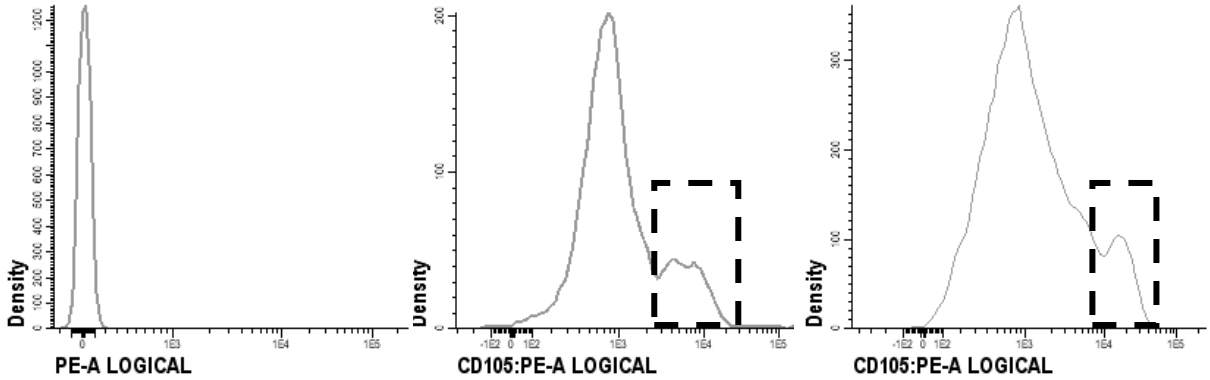


6. **Percentage CD117+ immature erythroid cells within erythroid fraction** defined as CD45$^{negative\text{-}to\text{-}dim}$ with SSC$^{low\text{-}to\text{-}int}$ CD117^{+} and CD71^{+}; figures 6A-F. Beware to exclude myeloid precursors. Since CD117^{+} erythroid cells may be hard to distinguish from myeloid progenitors an alternative might be to enumerate the CD105$^{bright+}$ immature erythroid cells within the erythroid fraction.
   An alternative strategy for enumerating CD117^{+} progenitors has been previously described by Matarraz et al. (Cytometry Part B: Clinical Cytometry 2010, 78B, pages 154–168).

7. **Percentage CD105^{+} immature erythroid cells within erythroid fraction** defined as: CD45$^{negative\text{-}to\text{-}dim}$ with SSC$^{low\text{-}to\text{-}int}$ CD105bright^{+} and CD71^{+} (beware not to include myeloid precursors (CD105^{-}CD117^{+}); figures 6A-F.



8. **CD105 expression.** CD105 expression level can be analyzed on immature CD105^{bright} cells. Examples in figures 7A, 7B and 7C. Immature CD105^{bright} erythroid cells in the MDS-example in figure 7C display increased expression as compared to NBM in figure 7B.



Technical information											
	center	A	B	C	D	E	E	F	G	H	I
						cohort 1	cohort 2				
Lysing solution		NH4Cl	NH4Cl	PharmLyse NH4Cl	PharmLyse NH4Cl	PharmLyse NH4Cl	PharmLyse NH4Cl	PharmLyse NH4Cl	FACS Lyse	NH4Cl	NH4Cl
Lysing time (minutes)		10	10	10	10	10	10	10	10	10	10
Temperature during lysing		RT	RT	RT	RT	RT	RT	RT	RT	RT	37
Application of fixative		no	no	no	no	no	no	no	no		no
Lyse-stain-wash or stain-lyse-wash?		LSW	LSW	LSW	LSW	LSW	LSW	LSW for CD235a else SLW	SLW	SLW	LSW
Flow cytometer		Canto-II BD Biosciences	Canto-II BD Biosciences	Calibur/Canto II BD Biosciences	Navios Coulter	Calibur BD Biosciences	Canto-II BD Biosciences	Canto-II BD Biosciences	Canto-II BD Biosciences	Navios Coulter	Canto-II BD Biosciences
X-color flow cytometry		6	6	4	10	4	8		8	4 and 5	8
Analysis software		Infinicyt	Infinicyt	CellQuestPro	Kaluza	CellQuestPro	Infinicyt	Kaluza	Infinicyt		Diva
Application of a nuclear dye?		no	no	no	no	no	no	not routinely	no	7-AAD	no
Antibodies											
CD45	Clone	2D1	2D1	2D1	J33	2D1	J33		OC515		HI30
CD45	Fluorochrome	PerCP	PerCP	PerCP	ECD	PerCP	KO	V500	PO		PO
CD45	Manufacturer	BD Biosciences	BD Biosciences	BD Biosciences	Beckman Coulter	BD Biosciences	Beckman Coulter	BD Biosciences	Cytognos		Invitrogen
CD71	Clone	LO 1,1	LO 1,1	1877	Ber-T9	Ber-T9	MA712		MA712	YDJ1.2.2	Lo1.1
CD71	Fluorochrome	APC	APC	FITC	FITC	FITC	APC-H7	FITC	APC-H7	FITC	FITC
CD71	Manufacturer	BD Biosciences	BD Biosciences	Beckman Coulter	Dako	Dako	BD Biosciences	BD Biosciences	BD Biosciences	BeckmannCoulter	BD Biosciences
CD235a	Clone	JC159	JC159	11E4B-7-6	11E4B-7-6- (KC16)	JC159	JC159				
CD235a	Fluorochrome	PerCP	PerCP	PerCP	PE	PE	PE	PE			
CD235a	Manufacturer	DAKO	DAKO	Beckman Coulter	Beckman Coulter	DAKO	DAKO	BD Biosciences			
CD36	Clone	CLB-IVC7	CLB-IVC7	CLB-IVC7	CLB-IVC7	CLB-IVC7	CLB-IVC7		FA6.152	FA6.152	CLB-IVC7
CD36	Fluorochrome	FITC	FITC	FITC	FITC	FITC	FITC	FITC	FITC	APC	FITC
CD36	Manufacturer	Sanquin	Sanquin	Sanquin	Sanquin	Sanquin	Sanquin	Beckman Coulter	Immunotech	Immunotech	Sanquin
CD117	Clone	104D2D1	104D2D1	104D2	104D2D1/104D2	104D2	104D2D1		IM3698		1G2
CD117	Fluorochrome	PE	PE	PE	PE/PC7	APC	PC7	PC7	PECy7		PE
CD117	Manufacturer	Beckman Coulter	Beckman Coulter	BD Biosciences	Coulter/BD Bio	BD Biosciences	Beckman Coulter	Beckman Coulter	Immunotech		Beckman Coulter
CD105	Clone		1G2				1G2		266		
CD105	Fluorochrome		PE				PE	PE	PE		
CD105	Manufacturer		Beckman Coulter				Beckman Coulter	Beckman Coulter	BD Bio		
Evaluation of expression level											
geo mean/median				geo mean	median			geo mean	geo mean	median	geomean 71/36 median 105
reference staining				lymphocytes	unstained cells				lymphocytes	unstained cells	
Evaluation of CD71 vs. CD235a pattern											
aberrancies are evaluated by				occurrence of CD71 dim	eyeballing			occurrence of CD71 dim	automated ref plots	occurrence of CD71 dim	

Technical information												
	center	J	J	K	L	M	N	O	P	Q	R	S
		tube 1	tube 2									
Lysing solution		NH4Cl	NH4Cl	PharmLyse NH4Cl	NH4Cl	NH4Cl	PharmLyse NH4Cl	FACS lysing solution	NH4Cl	NH4Cl	VersaLyse	NH_4Cl
Lysing time (minutes)		10	10	15	7	20	15	5 min	5	10	25	15 min
Temperature during lysing		RT	RT	RT	4	RT		RT	RT	RT	RT	RT (pre addition lysis solution kept at 37 C)
Application of fixative				no	no	yes	no	no	yes; 1%PFA	no		PFA
Lyse-stain-wash or stain-lyse-wash?		LSW	SLW	LSW	LSW or MNC	SLW		SLW	LSW	LSW	SLW	LSW
Flow cytometer		Canto-II BD Biosciences	Canto-II BD Biosciences	Canto-II BD Biosciences	Calibur BD Biosciences	Navios Coulter	Canto-II BD Biosciences	Canto-II BD Biosciences	Calibur BD Biosciences	Canto-II BD Biosciences	Navios Coulter	Calibur BD Biosciences
X-color flow cytometry		4	6	6	4	10 and 5		8		8	8	4
Analysis software		Infinicyt	Infinicyt	Diva	CellQuest	Kaluza	Kaluza	Diva and Infinicyt	WinList 6,0	DIVA	Kaluza	WinList
Application of a nuclear dye?		no	no	no	no	no	no	No	no	no	no	Select specimens
Antibodies												
CD45	Clone	2D1	2D1		2D1	J33	2D1	H130	2D1	H130	J33	2D1
CD45	Fluorochrome	PerCP	APC-H7	V450	PerCP	KO and PC7	PerCP	HV500	PerCP	HV500	KO	PerCP
CD45	Manufacturer	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BeckmannCoulter	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BeckmannCoulter	BD Biosciences
CD71	Clone	M-A712		MA712	M-A712	YDJ1.2.2		M-A712	G155-178	clone L01.1	YDJ1.2.2	M-A712
CD71	Fluorochrome	FITC		APC-H7	FITC	FITC	FITC	APC-H7	PerCP	APC	FITC	PE
CD71	Manufacturer	BD Biosciences		BD Biosciences	BD Bio	BeckmannCoulter	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BeckmannCoulter	BD Biosciences
CD235a	Clone	HIR2			GA-R2=HIR2	11E4B-7-6		JC159	10F7MN	10F7	11E4B-7-6	10F7
CD235a	Fluorochrome	PE		PerCP-Cy5.5	PE	PE	PE	PE	FITC	FITC	A750	FITC
CD235a	Manufacturer	e-Biosciences		BD Biosciences	BD Biosciences	BeckmannCoulter	Immunostep	DAKO	eBioscience	eBioscience	BeckmannCoulter	eBioscience
CD36	Clone		CB38		NL07	FA6.152		FA 6.152	G155-228	CB38	FA6.152	FA6.152
CD36	Fluorochrome		FITC	FITC	FITC	APC		FITC	APC	PE	APC	FITC
CD36	Manufacturer		BD Bio	BD Bio	eBioscience	BeckmannCoulter		Beckman-Coulter	BD Biosciences	BD Pharmingen	BeckmannCoulter	BD Biosciences
CD117	Clone	104D2	104D2		YB5.B8	104D2D1		A3C6E2	104D2	104D2	95C3	104D2
CD117	Fluorochrome	APC	APC	PE-CY7	PE	PC7		PE-Cy7	PerCP	PE-cy7	PerCPCY5.5	PE
CD117	Manufacturer r	BD Biosciences	BD Biosciences	BD Biosciences	eBioscience	BeckmannCoulter		Biolegend	BD Biosciences	BD Bio	BeckmannCoulter	BD Biosciences
CD105	Clone				SN6			na		266	IG2	SN6
CD105	Fluorochrome			APC	APC			na		PacBlue	PE	APC
CD105	Manufacturer				eBioscience			na		BD Biosciences	BeckmannCoulter	BD Biosciences
Evaluation of expression level												
geo mean/median		geo mean	geo mean	geo mean	geo mean			na	geo mean		geo mean	
reference staining		lymphocytes	lymphocytes	unstained cells	lymphocytes			na	unstained cells		lymphocytes	unstained cells
Evaluation of CD71 vs. CD235a pattern												
aberrancies are evaluated by		occurrence of CD71 dim		eyeballing	occurrence of CD71 dim	by eyeballing		eyeballing	increase in CV of CD71		eyeballing	difference or greater from normal

Supplementary tables and figures

Supplementary Table 1. **Comparison of results of the FC analysis of the erythroid lineage in the IMDSFlow dataset stratified by cohort**

	P MDS vs. PC	P MDS vs. NBM	P PC vs. NBM
Learning cohort			
relative %NEC	<0.001	<0.001	0.503
pattern CD71/CD235a	<0.001	<0.001	<0.001
%CD71dim	<0.001	<0.001	0.296
relative MFI of CD71	<0.001	<0.001	0.619
relative CV of CD71	<0.001	<0.001	0.485
relative MFI of CD36	0.001	<0.001	0.139
relative CV of CD36	<0.001	<0.001	0.020
relative %CD117 progenitors	0.711	0.787	0.536
relative %CD105 progenitors	0.399	0.179	0.355
relative MFI of CD105	0.136	0.002	0.008
Validation cohort			
relative %NEC	0.368	0.022	0.106
pattern CD71/CD235a	<0.001	<0.001	0.284
%CD71dim	0.092	<0.001	<0.001
relative MFI of CD71	0.180	0.284	0.498
relative CV of CD71	0.002	<0.001	0.639
relative MFI of CD36	0.145	0.014	0.101
relative CV of CD36	<0.001	<0.001	0.254
relative %CD117 progenitors	0.910	0.148	0.010
relative %CD105 progenitors	0.162	0.217	0.805
relative MFI of CD105	0.304	0.168	0.715

Note: Collected data were normalized against the median value determined in the set of normal bone marrow samples, except for aberrant pattern of CD71 vs. CD235a and %$CD71^{dim}$. P-values represent the results of the comparison performed by the Kruskal Wallis test, except for aberrant pattern of CD71 vs. CD235a which was analyzed by the Fisher's exact test. Abbreviations: dim: diminished; CV: coefficient of variation; MDS: myelodysplastic syndromes; NBM: normal bone marrow samples; NEC: nucleated erythroid cells; PC: pathological controls.

Supplementary Table 2A. **Cut-offs applied for evaluation of results from the FC analysis of erythroid markers in the learning cohort**

	10th percentile	90th percentile	# of PC cases*	# of NBM cases
relative %NEC	**-**	**268%**	238	139
%CD71^{dim}	**-**	**17%**	250	129
relative MFI of CD71	**45%**	**-**	250	126
relative CV of CD71	**-**	**133%**	165	88
relative MFI of CD36	**53%**	**-**	203	124
relative CV of CD36	**-**	**145%**	177	92
relative %CD117 progenitors	**37%**	**222%**	180	122
relative %CD105 progenitors	**50%**	**184%**	52	59
relative MFI of CD105	**52%**	**113%**	70	47

Note: : Cut-off values represent the 10th and 90th percentiles of results for erythroid markers among pathological controls in the learning cohort. The number of pathological control (PC) cases that were available to calculate cut-off values are displayed (*). Most values (except for %CD71^{dim}) are expressed as ratio to the median value determined in the set of normal bone marrow samples. The utmost right column displays the number of normal bone marrow (NBM) cases that were available to calculate these median values. Abbreviations: CV: coefficient of variation; dim: diminished; MFI: mean fluorescence intensity; NEC: nucleated erythroid cells.

Example on how to translate these reference ranges for application in a single center:
In case a sufficient amount of data is present regarding a large variation of pathological controls, 10th and 90th percentiles calculated from a center's own cohort may be applied when comparable to the herein described reference values.
Otherwise, collect data on an appropriate amount of normal bone marrow samples (min. 10) and determine median values to calculate the cut-offs for the parameters in the erythroid score.
For instance, the median CV value of CD71 in your set of normal bone marrow samples is 67 and the median percentage of CD117^{+} erythroid progenitors in the erythroid compartment is 8%. Then the reference values for CD71 CV is: 133/100*67=89; a CD71CV>89 should be considered increased. "133" is the 90th percentile for CD71 CV from supplementary table 2A.
Similarly for %CD117^{+}: lower cut-off 37/100*8=3.0 and highest cut-off 222/100*8=17.8; i.e. a CD117+ percentage (erythroid compartment) below 3.0% or above 17.8% should be considered aberrant.

Supplementary Table 2B. **Comparison of 10th and 90th percentiles of selected erythroid FC markers stratified by cohort**

	Learning cohort		Validation cohort	
	10th percentile	90th percentile	10th percentile	90th percentile
relative MFI of CD71	**45%**	**-**	**49%**	**-**
relative CV of CD71	**-**	**133%**	**-**	**147%**
relative CV of CD36	**-**	**145%**	**-**	**134%**
relative %CD117 progenitors	**37%**	**222%**	**31%**	**172%**

Supplementary table 3. **Percentage of flow cytometric aberrancies in the erythroid lineage among MDS and controls stratified by cohort**

	NBM	PC	MDS
Learning cohort			
relative %NEC	2.9	10.1	32.1
pattern CD71-CD235a	3.7	17.3	64.9
%CD71^{dim}	0.8	10.0	31.5
relative MFI of CD71	4.0	10.0	27.8
relative CV of CD71	4,5	10.3	45.5
relative MFI of CD36	0.8	10.3	25.8
relative CV of CD36	0.0	10.2	30.1
relative %CD117 progenitors	8.2	19.4	33.8
(decreased)	(4.9)	(10.0)	(15.2)
(increased)	(3.3)	(9.4)	(18.6)
relative %CD105 progenitors	6.7	20.8	48.4
(decreased)	(3.3)	(7.8)	(26.6)
(increased)	3.3	(13.0)	(21.9)
relative MFI of CD105	29.8	22.5	58.7
(decreased)	(4.3)	(11.3)	(38.1)
(increased)	(25.5)	(11.3)	(20.6)
Validation cohort			
relative %NEC	2.1	18.7	23.0
pattern CD71-CD235a	4.8	16.7	54.5
%CD71^{dim}	4.8	20.6	19.8
relative MFI of CD71	2.3	8.9	10.6
relative CV of CD71	2.1	16.7	27.3
relative MFI of CD36	6.5	14.6	27.4
relative CV of CD36	0.0	5.3	19.7
relative %CD117 progenitors	2.2	20.1	40.8
(decreased)	(0.0)	(13.4)	(22.4)
(increased)	(2.2)	(6.7)	(18.4)
relative %CD105 progenitors	2.8	24.2	45.7
(decreased)	(0.0)	(10.1)	(18.5)
(increased)	(2.2)	(14.1)	(27.2)
relative MFI of CD105	23.7	47.0	55.8
(decreased)	(0.0	(7.6)	(7.8)
(increased)	(23.7	(39.4)	(48.1)

Numbers represent percentages of cases that scored abnormal when compared with the defined cut-offs for a particular marker (*Supplementary Table 2.*). Abbreviations: dim: diminished; CV: coefficient of variation; MDS: myelodysplastic syndromes; NBM: normal bone marrow samples; NEC: nucleated erythroid cells; PC: pathological controls.

Supplementary Table 4. **Correlation of age and FC markers of the erythroid lineage in normal bone marrow samples within the learning cohort**

	Spearman's Rho	P
relative %NEC	0.12	0.179
% aberr. pattern CD71/CD235a	0.08	0.366
%$CD71^{dim}$	0.13	0.153
relative MFI of CD71	-0.20	0.032
relative CV of CD71	0.17	0.148
relative MFI of CD36	0.21	0.026
relative CV of CD36	-0.01	0.925
relative %CD117 progenitors	-0.05	0.640
relative %CD105 progenitors	0.30	0.136
relative MFI of CD105	**-0.55**	**<0.001**

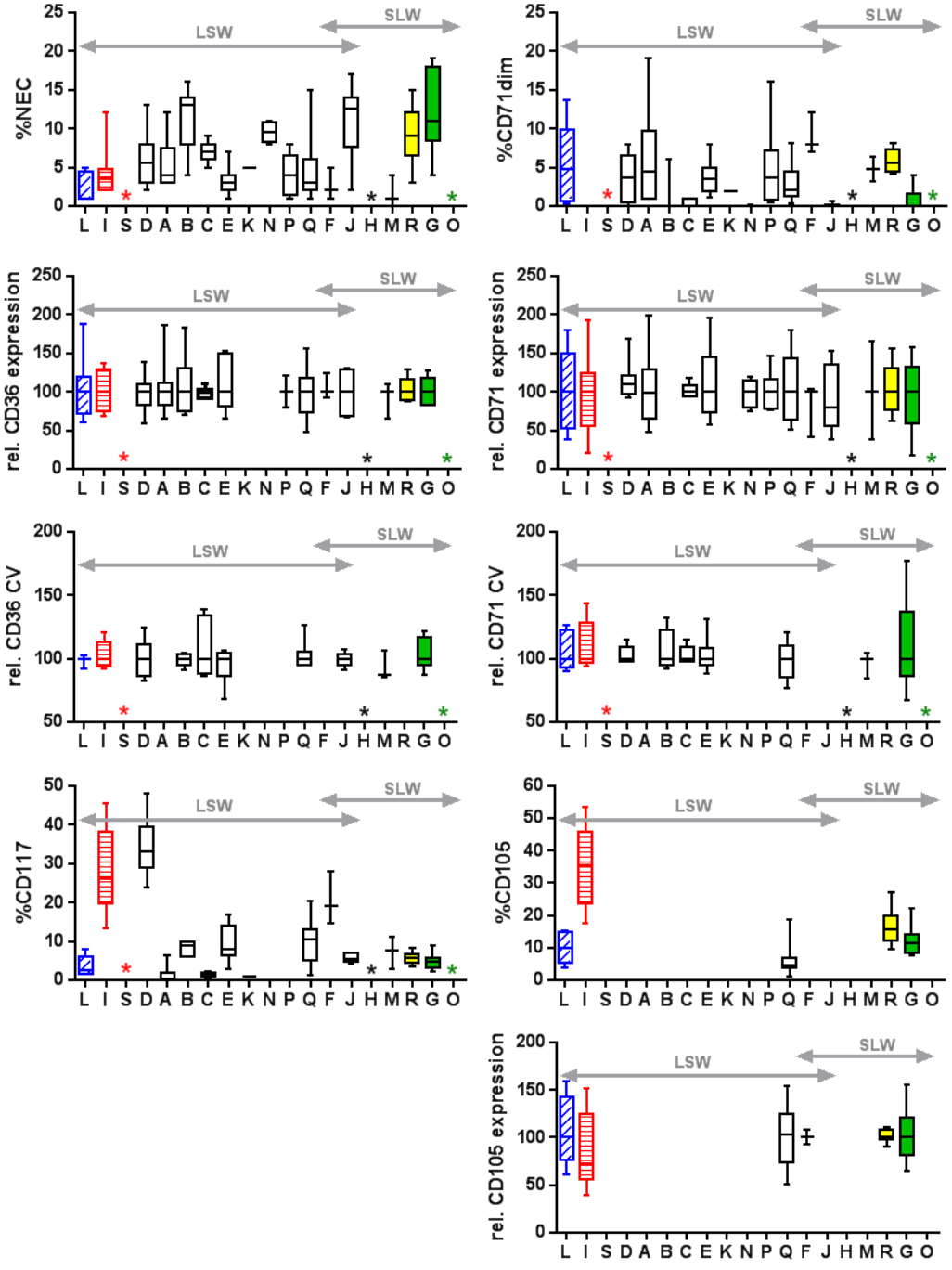
Abbreviations: aberr.: aberrant; CV: coefficient of variation; MFI: mean fluoresce intensity; NEC: nucleated erythroid cells

Supplementary Table 5. **Results of the FC erythroid dysplasia score among MDS cases and controls stratified per cohort.**

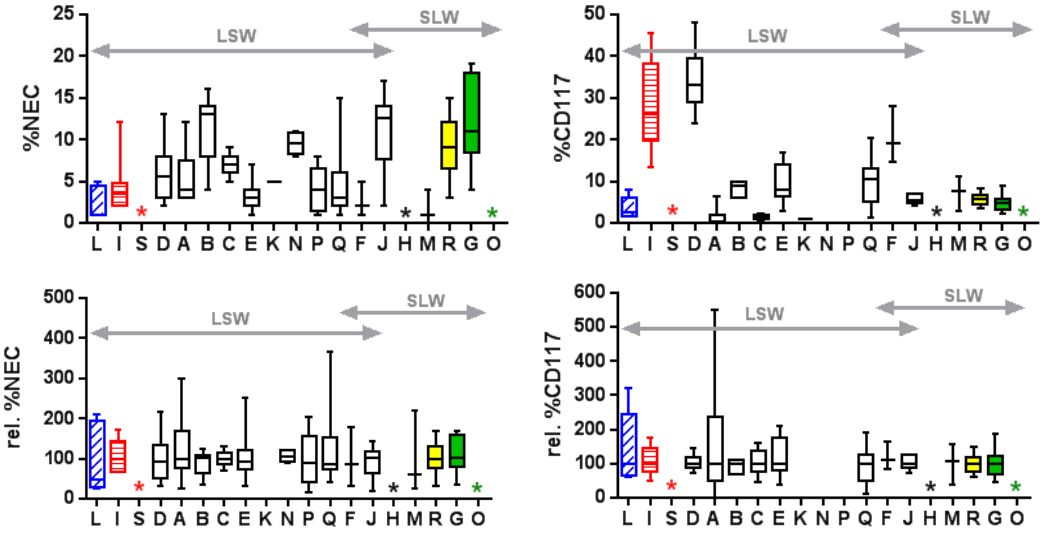
	Learning Cohort		Validation Cohort		Combined Cohorts	
	FC erythroid dysplasia score ≥5	# of cases	FC erythroid dysplasia score ≥5	# of cases	FC erythroid dysplasia score ≥5	# of cases
Subset						
normal	**3**	2/79	**0**	0/42	**2**	2/121
pathological controls	**10**	15/153	**8**	8/106	**9**	23/259
MDS	**33**	39/119	**24**	22/93	**29**	61/212
MDS subcategories						
RCUD	**33**	5/15	**43**	3/7	**41**	9/22
RARS(-t)	**57**	8/14	**43**	3/7	**52**	11/21
RCMD	**30**	24/79	**22**	12/58	**26**	36/137
del(5q)	**13**	1/8	**33**	1/3	**27**	3/11
MDS not specified			**6**	1/16	**6**	1/16
Pathological control subcategories						
iron deficiency anemia	**6**	1/18	**9**	1/11	**7**	2/29
anemia in chronic disease[†]	**10**	3/29	**20**	1/5	**12**	4/34
vitamin B12/folic acid deficiencies	**0**	0/6	**14**	1/7	**8**	1/13
anemia in auto-immune diseases[†]	**0**	0/5			**17**	1/6
anemia due to renal failure		1/3		0/2	**20**	1/5
anemia other*	**0**	0/5	**20**	1/5	**10**	1/10
cytopenia associated with marrow infiltration		1/3	17	1/6	**22**	2/9
cytopenia induced by chemotherapy or medication or post-SCT	**0**	0/7	**0**	0/6	**0**	0/13
ITP or neutropenia or auto immune cytopenia NOS	**14**	3/21	**0**	0/6	**15**	3/27
reactive conditions or cytopenia induced by infections	**28**	5/18	**0**	0/5	**22**	5/23
normal bone marrow (peripheral cytopenia NOS)	**20**	1/5			**20**	1/5
other subcategories	**0**	0/20	**0**	0/22	**0**	0/42
inconclusive	**0**	0/11	**0**	0/7	**0**	0/18
non clonal cytopenia NOS			**0**	0/18	**0**	0/18

Note: Data represent percentage of subjects with an FC erythroid score of ≥2 and the actual amount of 'positive' cases per available cases. Only data of subsets with five or more cases are depicted; diagonally marked cells represent data not available or reliable (i.e., missing or only small data sets (<5 cases)). [†]the subset "anemia in chronic disease" comprises iron incorporation disorders, chronic bowel diseases, diabetes, etc.; "anemia in auto-immune diseases" comprises AIHA, AITP, Rheumatoid Arthritis, SLE, etc.; and the subset "anemia other" contains among others cases of normocytic anemia, anemia unexplained, etc. NOS: not otherwise specified.

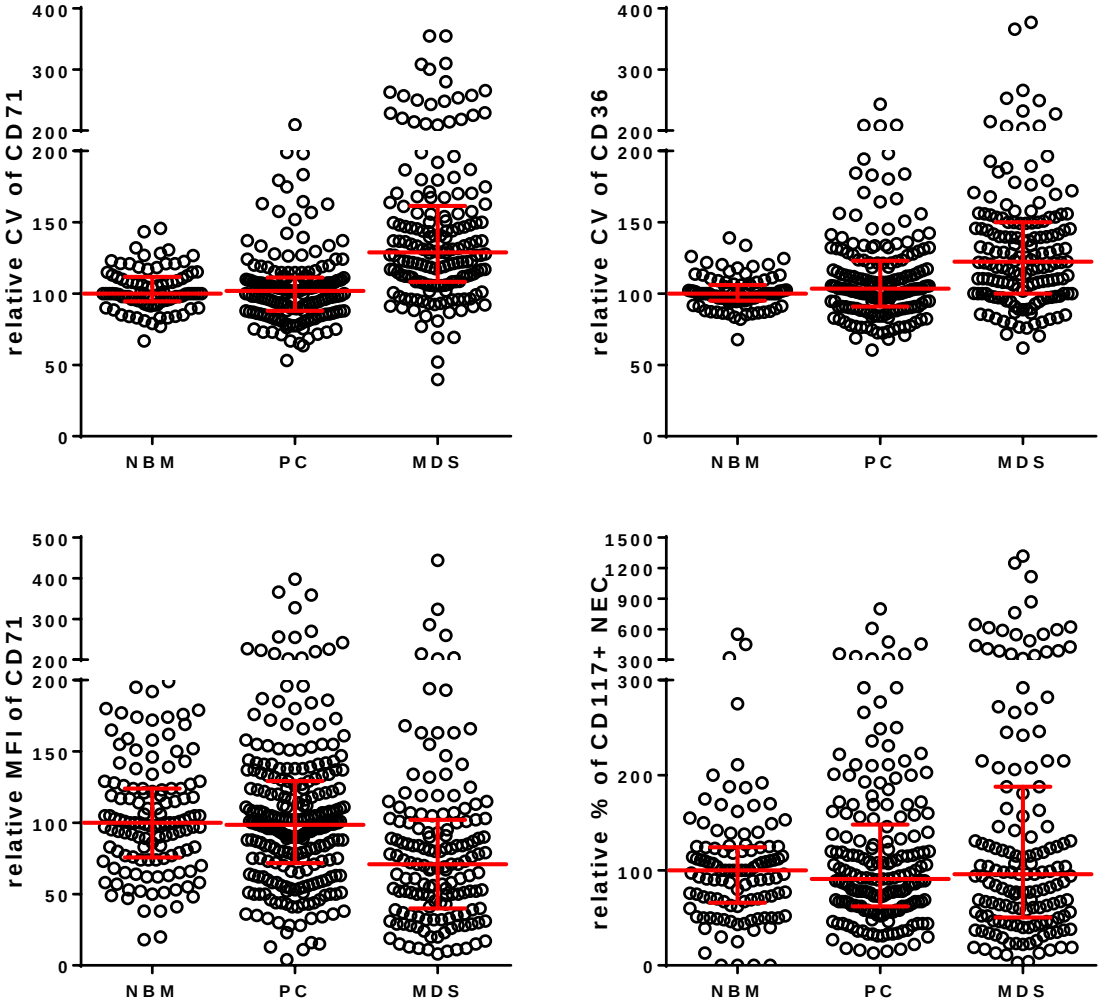
Supplementary Figure 1. **Results for erythroid markers in normal bone marrow samples analyzed by FC within different centers (learning cohort).** Data are presented as boxplots with median and range. Depicted erythroid markers are percentage of nucleated erythroid cells (NEC), relative (rel.) expression of CD71, rel. CV of CD71 expression, rel. expression of CD36, rel. CV of CD36 expression, subset of $CD71^{dim}CD235a^{+}$ cells in CD71-CD235a pattern, rel. expression of CD105, percentage of $CD117^{+}$ cells in the erythroid compartment and percentage of $CD105^{+}$ cells in the erythroid compartment. The different centers were anonymized (A to S) in order of data entry in the cohort. Open boxes reflect application of ammonium chloride as lysing procedure (PharmLyse, BD Biosciences or home-made; yellow boxes: Versalyse (BeckmanCoulter); green boxes FACSLyse (BD Biosciences); blue-edged boxes indicate lysis at 4ºC, red-edged boxes at 37ºC and black boxes at room temperature. Centers M and R use longest duration of incubation with lysing solution 20 and 25 minutes, respectively. No data for normal bone marrow samples were are available for centers H and O; center S only entered data in the validation cohort. Asterisks indicate the procedures in these centers; red: ammonium chloride at 37ºC; black: ammonium chloride at room temperature; green: FACS lyse at room temperature. Abbreviations: LSW: lyse-stain-wash; SLW: stain-lyse-wash. Centers F and J applied LSW in a tube containing CD235a and SLW in other tubes.



Supplementary Figure 2. **Results for erythroid markers in normal bone marrow samples analyzed by FC within different centers (learning cohort).** Data are presented as boxplots with median and range. Depicted erythroid markers are percentage of nucleated erythroid cells (NEC) and the percentage of $CD117^+$ cells in the erythroid compartment before and after normalization (upper and lower panel, respectively). The different centers were anonymized (A to S) in order of data entry in the cohort. Open boxes reflect application of ammonium chloride as lysing procedure (PharmLyse, BD Biosciences or home-made; yellow boxes: Versalyse (BeckmanCoulter); green boxes FACSLyse (BD Biosciences); blue-edged boxes indicate lysis at 4ºC, red-edged boxes at 37ºC and black boxes at room temperature. Centers M and R use longest duration of incubation with lysing solution 20 and 25 minutes, respectively. No data for normal bone marrow samples were are available for centers H and O; center S only entered data in the validation cohort. Asterisks indicate the procedures in these centers; red: ammonium chloride at 37ºC; black: ammonium chloride at room temperature; green: FACS lyse at room temperature. Abbreviations: LSW: lyse-stain-wash; SLW: stain-lyse-wash. Centers F and J applied LSW in a tube containing CD235a and SLW in other tubes.



Supplementary Figure 3. **Comparison of results of analysis of the proposed FC-markers for erythroid dysplasia in MDS and controls (learning cohort).**
Data are presented as scatterplots with median and interquartile regions (indicated in red) of normal bone marrow samples (NBM), pathological controls (PC) and MDS. Median and range are summarized in the table below. Depicted erythroid markers are the relative CV of CD71 and CD36, the relative MFI of CD71 and the percentage of $CD117^{+}$ nucleated erythroid cells (NEC) within the erythroid compartment after normalization against NBM samples. Figures demonstrate increased CVs of CD36 and CD71 in MDS, decreased MFI of CD71 and a broader range of the percentage CD117+ erythroid progenitors as compared to controls. Accompanying P-values are depicted in Supplementary Table 1.



Learning cohort	NBM	PC	MDS
relative CV of CD71	100 (67-146)	102 (53-209)	129 (69-355)
relative CV of CD36	100 (68-139)	104 (60-243)	122 (62-377)
relative MFI of CD71	100 (18-199)	99 (4-398)	71 (8-444)
relative %CD117 progenitors	100 (0-550)	91 (13-800)	96 (3-1319)

Supplementary Figure 4. **Correlation of age and FC markers of the erythroid lineage in normal bone marrow samples within the learning cohort and the combination of learning and validation cohorts.** Scatterplots are displayed for markers that showed a significant relation with age in the learning cohort (panel A): relative MFI of CD36, CD71 and CD105. The results of the same markers in the combined learning and validation cohorts are displayed in Panel B. Spearman's Rho and p-values are indicated in the plots as no: Spearman's Rho -0.2–0.2; poor: Spearman's Rho -0.2– -0.5; moderate-to good: Spearman's Rho -0.5– -0.7; and n.s.: p>0.05; *: p<0.05; **: p<0.001, respectively.