Validated single-tube multiparameter flow cytometry approach for the assessment of minimal residual disease in multiple myeloma

Multiple myeloma (MM) is a plasma cell (PC) malignancy that typically affects elderly patients. The outcome in terms of overall survival (OS) is heterogeneous, depending on the biology of the disease itself, prognostic factors and responses to therapy.[1] The treatment landscape for MM has improved greatly,[2] leading to deeper remissions and prolonged progression-free survival (PFS) and OS.[3] These advances created the need for highly sensitive means to detect minimal residual disease (MRD), which is valuable for the evaluation of depth of responses and therapy monitoring.[4] The aim now is to use MRD as a surrogate endpoint, rather than later endpoints (i.e., PFS and/or OS) which need long periods of time to mature, until they may show meaningful differences resulting from the allocation of novel therapies.[5] Multiparameter flow cytometry (MFC) enables detection of residual aberrant PC (aPC) with high sensitivity.[6] Currently, there are several MFC options available, including EuroFlow and other analyses.[4,6,7] EuroFlow uses a two-tube, eight-color assay that ensures the currently aimed sensitivity of 10^{-5}: the sample must be divided into two tubes and the

Table 1. Characteristics of the patients from whom bone marrow samples were taken at initial diagnosis, at disease progression or under treatment, overall and divided according to whether they were studied with the six- or eight-color panel.

	Total cohort (n=163)[a]		6-color (n=112)		8-color (n=63)	
	N. (%)	Median (range)	N. (%)	Median (range)	N. (%)	Median (range)
Stage						
MM	138 (84)		93 (83)		57 (91)	
ID/PD	73 (45)		51 (45)		23 (37)	
Post-CTx	10 (6)		3 (3)		7 (11)	
Post-auto/allo-SCT	55 (33)		39 (35)		27 (43)	
SMM/MGUS	25 (16)		19 (17)		6 (9)	
Sex						
Female	72 (44)		47 (42)		29 (46)	
Male	91 (56)		65 (58)		34 (54)	
Age (years)						
ID		61 (28-85)		60 (28-85)		63 (32-84)
Sampling		65 (29-86)		64 (29-85)		66 (32-84)
Durie & Salmon (ID)						
I	39 (24)		29 (26)		10 (16)	
II	36 (22)		21 (19)		20 (32)	
III	88 (54)		62 (55)		33 (52)	
A/B	142 (87) / 21 (13)		100 (89) / 12 (11)		54 (86) / 9 (14)	
International Staging System (ID)						
I	58 (35)		42 (37)		19 (30)	
II	43 (26)		29 (26)		17 (27)	
III	63 (39)		41 (37)		27 (43)	
MM type						
IgG	105 (64)		75 (67)		38 (60)	
IgA	25 (15)		14 (12)		12 (19)	
Light chain only	34 (21)		23 (21)		13 (21)	
Light chain						
Kappa	106 (65)		75 (67)		38 (60)	
Lambda	57 (35)		37 (33)		25 (40)	
MM progression						
Yes	29 (18)		26 (23)		5 (8)	
No	134 (82)		86 (77)		58 (92)	
Vital status						
Dead	16 (10)		15 (13)		1 (2)	
Alive	147 (90)		97 (87)		63 (98)	

MM: multiple myeloma; ID: initial diagnosis; PD: progressive disease; post-CTx: after standard chemotherapy; post-ASCT: after autologous stem cell transplantation; post-alloSCT: after allogeneic stem cell transplantation; SMM: smoldering multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance. [a]Some patients were assessed more than once during the period of sampling. Every patient was only counted once for each cohort. The number of both cohorts together is higher than that of the entire cohort because some patients were assessed sequentially.

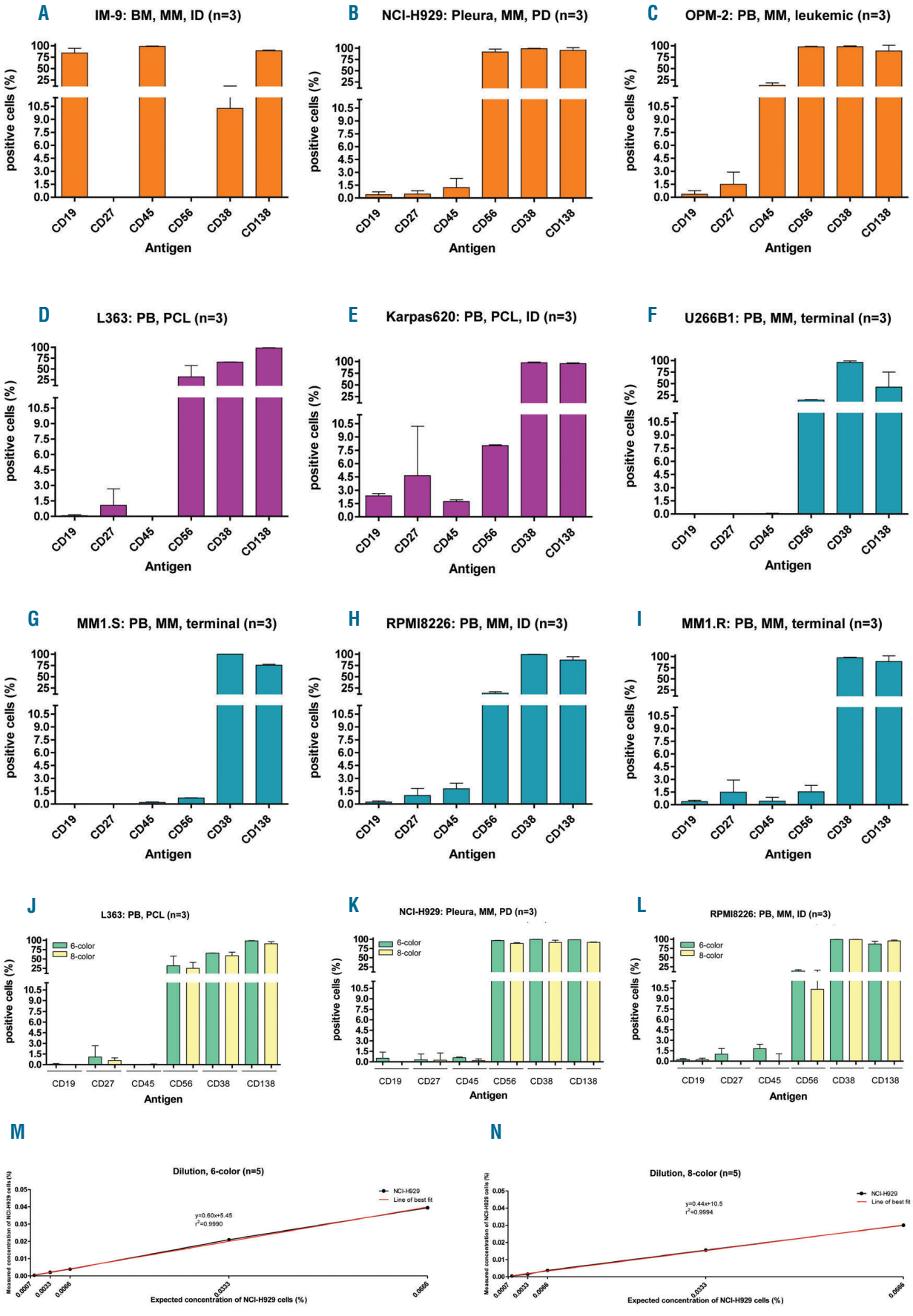

A
IM-9: BM, MM, ID (n=3)
B
NCI-H929: Pleura, MM, PD (n=3)
C
OPM-2: PB, MM, leukemic (n=3)
D
L363: PB, PCL (n=3)
E
Karpas620: PB, PCL, ID (n=3)
F
U266B1: PB, MM, terminal (n=3)
G
MM1.S: PB, MM, terminal (n=3)
H
RPMI8226: PB, MM, ID (n=3)
I
MM1.R: PB, MM, terminal (n=3)
positive cells (%)
Antigen
CD19
CD27
CD45
CD56
CD38
CD138
J
L363: PB, PCL (n=3)
K
NCI-H929: Pleura, MM, PD (n=3)
L
RPMI8226: PB, MM, ID (n=3)
6-color
8-color
M
Dilution, 6-color (n=5)
y=0.60x+5.45
r²=0.9990
N
Dilution, 8-color (n=5)
y=0.44x+10.5
r²=0.9994
NCI-H929
Line of best fit
Measured concentration of NCI-H929 cells (%)
Expected concentration of NCI-H929 cells (%)

Figure 1 (previous page). Assay validation: analytical specificity, staining stability and sensitivity of the six-color and eight-color panel. (A-I) Nine different multiple myeloma cell lines, IM-9 (A), NCI-H929 (B), OPM-2 (C), L363 (D), Karpas620 (E), U266B1 (F), MM1.S (G), RPMI8226 (H), and MM1.R (I), were analyzed for their expression of CD19, CD27, CD45, CD56, CD38 and CD138 in the six-color panel in three biological replicates. All cell lines expressed low levels of CD19 and CD27 and high levels of CD138 and CD38. The expression of CD45 and CD56 was quite heterogeneous. According to their *in vitro* proliferative behavior: IM-9, NCI-H929 and OPM-2 (A-C) proliferate in a suspension cluster formation (orange graphs), L363 and Karpas620 are suspension cells (D, E; violet graphs) and U266B1, MM1.S, RPMI8226 and MM1.R are semi-adherent (F-I; light blue graphs). In none of the three proliferative groups was a characteristic antigen expression pattern observed. (J-L) Three different multiple myeloma cell lines were investigated using both panels and the expression of the antigens in common was compared in order to assess any differences in extracellular staining due to additional intracellular staining in the eight-color panel. The expression of the antigens CD19, CD27, CD45, CD56, CD38 and CD138 was similar. Thus, the fixation and permeabilization steps in the eight-color panel assay had no impact on the expression of the surface markers. (M, N) The multiple myeloma cell line NCI-H929 was diluted into human peripheral blood mononuclear cells from a healthy individual in five defined concentrations. The expected linearity could be demonstrated with an r^2=0.9990 for the six-color panel (M) and r^2=0.9994 for the eight-color panel (N). Graphs show the mean ± standard deviation of three biological replicates (A-L) and mean of five biological replicates (M, N), unpaired *t*-test (J-L) and linear regression (M, N). BM: bone marrow; MM: multiple myeloma; CD: cluster of differentiation; ID: initial diagnosis; PD: progressive disease; PB: peripheral blood; PCL: plasma cell leukemia.

assay requires special equipment, which increase time, efforts and costs.[4,6] Therefore, leading US groups established an alternative single-tube, ten-color method: in a head-to-head comparison of this assay with the EuroFlow assay, the sensitivity was similar but there were substantial reductions in time and costs (*Online Supplementary Figure S1*).[8,9] Since most MFC panels remain cost-intensive and need ten-color machines (*Online Supplementary Figure S1*), they may not be affordable for every laboratory.[8–10] Furthermore, these MFC panels are not routinely available for MM patients in and outside clinical trials.

On this background, the current study was initiated, with the purpose of developing a cost-effective, simple, easily usable MRD flow panel for MM patients at tertiary centers, as an alternative to more cost-intensive panels.[6,11] It was aimed to meticulously validate this MFC panel in MM cell lines first and then in routinely bone marrow (BM)-punctured MM patients, both in and outside clinical trials, to reflect everyday MM cohorts.

BM samples were lysed and directly stained with the six cell-surface antibodies CD138, CD38, CD45, CD56, CD27, and CD19 (*Online Supplementary Table S1*) included in both six- and eight-color panels. The eight-color panels additionally included the intracellular antibodies kappa (κ) and lambda (λ) (*Online Supplementary Table S1*). Initially, MM cell lines were assessed with both the six- and eight-color panels. For all analyses, $3x10^6$ events were acquired. Patients' samples were defined MRD negative (MRD^-) if the total nucleated cells contained $<10^{-5}$ aPC. aPC were typically $CD138^+CD38^+CD56^{+/-}CD45^-CD19^{-/+}CD27^{-/+}$ using the six-color panel (*Online Supplementary Figure S2A*) and, using the eight-color panel, monoclonality for κ or λ was determined (*Online Supplementary Figure S2B*). Normal PC (nPC) were typically $CD138^+CD38^+CD56^{-/+}CD45^+CD19^{-/+}CD27^+$ and polyclonal for κ and λ (details of the methods are available in the *Online Supplementary Material*).

The levels of expression of the six cell-surface antigens in nine different MM cell lines (Figure 1A-I) were consistent with those in prior reports (*Online Supplementary Table S2*). Side-by-side assessment of three MM cell lines using both six- and eight-color panels (Figure 1J-L) showed no differences between the expression levels of the six cell-surface antigens in common. This confirmed that the cell-surface staining was not significantly affected by the additional intracellular staining in the eight-color panel. In corroboration with the absence of aPC in BM samples of healthy individuals, this validated the analytical specificity of both the six- and eight-color panels. To determine the sensitivity of both panels, MM cell lines were spiked into human peripheral blood mononuclear cells in five linear concentrations down to $<10^{-5}$ and detected in a total of $3x10^6$ events (Figure 1M, N). The limit of detection was 30 cells for both panels, resulting in a sensitivity of 10^{-5}, as reported.[11] Further validation analyses were conducted to assess the stability and robustness of both panels and to optimize the protocol according to consensus recommendations (*Online Supplementary Figure S3*).[11]

In-depth validation analyses were conducted thereafter to standardize the MFC protocol for the detection of aPC in MM patients.[11] Notably, phenotypes differed substantially between MM patients, but the two panels were equally robust and reliably detected aPC and nPC in patients' samples. An advantage of the six-color panel was the straightforward staining procedure. In 2/11 (18%) phenotypically uncertain cases (in which aPC appeared close to nPC, despite high BM infiltration rates *via* histology), the extracellular staining *via* the six-color panel was less precise, whereas the eight-color panel, including κ and λ, offered an additional tool for assessing clonality and ensured reliable detection of aPC. Thus, the eight-color assay was determined best for MRD detection. Moreover, it was pertinent that the sensitivity of 10^{-5} with a MRD cut-off of $<10^{-5}$ (as achieved in other MFC panels[6,11]) was also obtained in our in-depth MCF analyses.

In 205 BM samples from patients, aPC were detected and could be distinguished from nPC with both panels. The characteristics of the entire cohort of patients (n=163) and the cohorts of patients tested by the six- and eight-color panels showed age, gender and disease characteristics very typical for tertiary centers and comparability of both cohorts (Table 1). Since MM cell line results and patients' characteristics in the six- and eight-color analyses were comparable, combined analysis of both cohorts was performed.

A total of 74 samples at initial diagnosis and 101 samples under treatment were assessed with both panels. When comparing patients with MM (n=49) to those in premalignant stages (n=25), namely monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM),[12] significantly higher numbers of aPC were observed in MM patients than in those with MGUS/SMM (P<0.001) (Figure 2A), illustrating increasing aPC associated with patients' disease status (*Online Supplementary Figure S2A, B*). This increase of aPC from premalignant disease stages to symptomatic MM was described previously,[13] and supports the reliability of our gating strategy and MCF assay.

Another focus was to determine the MRD status after antimyeloma treatment. The samples in the MRD cohort (n=101) were analyzed for MRD at a sensitivity of 10^{-5}, which was achieved for 94% of the samples (Figure 2B). Based on the MRD cut-off ($<10^{-5}$), 26% (n=26) reached

MRD⁻, whereas 74% (n=75) remained MRD⁺. Expectedly, the number of aPC was significantly higher in MRD⁺ cases than in MRD⁻ patients (P<0.0001) (Figure 2C), in line with different treatment responses. The median time from the start of standard treatment or autologous/allogeneic stem cell transplantation (SCT) until MRD determination was fairly short, being 45 days (range, 27-5949), and was comparable for MRD⁻ and MRD⁺ patients (42 *vs.* 47 days, respectively).

Age, gender and MM characteristics were again similar in both the MRD⁺ and MRD⁻ groups (Table 2). None of the MM characteristics, i.e. BM infiltration at initial diagnosis, stage, cytogenetics or MM type, were predictive for achievement of MRD⁻ after treatment. This was observed for both the cohorts tested with the six-color panel and the one tested with the eight-color panel (*Online Supplementary Table S3*), again confirming the comparability of the two panels. Analysis of the influence of treatment intensity on MRD levels showed that 99% of patients in the MRD⁻ group had received intensive treatment (autologous or allogeneic SCT) *vs.* 87% in the MRD⁺ group. At the time of MRD determination, no cytogenetic aberrations were present in the MRD⁺ group and those patients re-assessed by fluorescence *in situ* hybridization (FISH) (56%) had normal karyotypes, whereas 16% of the MRD⁺ patients had persistent cytogenetic aberrations by FISH (high risk 4% *vs.* standard risk 12%).

Association of MRD levels with remission status revealed that complete remissions and very good partial remissions were reached in 88% of the MRD⁻ cohort *vs.* 76% in the MRD⁺ group. In the MRD⁻ cohort, only 12% showed partial remission and none had stable disease, whereas in the MRD⁺ group, 21% had a partial remission and 3% had stable disease according to the International Myeloma Working Group response definition.[14]

Since MRD⁻ is associated with improved PFS and OS,[5] these outcomes were also assessed: the median PFS was 17.7 months in the MRD⁺ group and was not reached in the MRD⁻ group (P=0.2408) (Figure 2D). Only minor differences were observed in OS (Figure 2E). The median time of observation was short, being 7.9 months (range, 1-24; from the first patient in until the stopping date: June 19, 2019). MRD⁻ patients with standard-risk cytogenetics showed the most favorable PFS (median not reached) (*Online Supplementary Figure S4A*). Of note, MRD⁻ patients with high-risk cytogenetics had a more favorable PFS than MRD⁺ standard-risk and high-risk patients (median PFS: not reached *vs.* 17.9 and 15.4 months, respectively) (*Online Supplementary Figure S4B*).

According to the MRD cut-off ($<10^{-5}$), 26% of patients reached MRD⁻ and 74% remained MRD⁺ in this study, similar to the rates in the study by Campbell *et al.*, in which 28% of the entire cohort were MRD⁻ 3 months after autologous SCT.[15] Our MRD⁻ rate of ~30% was also comparable to that of other analyses, but somewhat lower than the 59% in much quoted studies,[16–18] most likely because of our early MRD determination, while others analyzed MRD status later (3-9 months after the start of treatment or SCT).[16] Our MRD determination after standard treatment or SCT reflected the routine BM evaluation schedule at our and other international centers, e.g., at day 30-60 after SCT. Additionally, the percentages of MRD⁻ patients have often been calculated solely from patients reaching complete remission, not from consecutive patients.[17] In our study, 88% of patients in the MRD⁻ group were in complete or very good partial remission *vs.* 76% in the MRD⁺ cohort, suggesting that assessment of only patients in complete remission for MRD levels underestimates MRD⁻ rates. These findings were similar to others,[19] but compared to reports in the literature, fewer patients were in complete remission (20% in our analysis *vs.* ~50% in other analyses), primarily due to the shorter time between start of standard treatment or SCT and MRD determination and the limited follow-up in our study. Only 1% in our MRD⁻ group progressed, whereas 21% in the MRD⁺ group did so, confirming the validity of the MRD⁻ achievement and correlation with treatment response.[18]

In addition to the analysis of aPC, we also analyzed nPC: the numbers of nPC were significantly higher in SMM/MGUS than in MM samples at initial diagnosis (P=0.0355) (Figure 2F), suggesting a decline of nPC with disease progression from MGUS/SMM to MM. The significantly higher number of nPC in SMM/MGUS samples than in MM samples at initial diagnosis may therefore serve as a prognostic factor for progression from pre-MM stages into symptomatic MM, because the presence of nPC plays a pivotal role in maintaining the normal BM PC environment.[13]

The numbers of nPC were significantly higher in healthy individuals (n=13) than in MM patients at initial diagnosis (n=49; P=0.008), at disease progression (n=30; P=0.006) and under treatment (n=101; P=0.0067) (Figure 2G). Of interest, the numbers of nPC appeared significantly higher in MRD⁺ *vs.* MRD⁻ samples (P=0.0169) (Figure 2H).

To assess this after treatment in more detail, BM samples were compared in three different treatment groups: (i) after standard chemotherapy (n=11); (ii) after autologous SCT (n=77); and (iii) after allogeneic SCT (n=13). Again, nPC numbers were significantly higher in samples from healthy individuals than in those from patients after standard chemotherapy (P=0.0077), autologous SCT (P=0.0061) or allogeneic SCT (Figure 2I), although they were higher in the latter two groups than in the samples from patients after standard chemotherapy. It is possible that nPC numbers were significantly higher in the MRD⁺ group because of less suppression of BM cells.[13] Higher nPC numbers after autologous or allogeneic SCT may also indicate a reversion to a normal BM status in MM patients and that nPC in MM after SCT "reset" to similar levels as in MGUS patients or healthy individuals.[13]

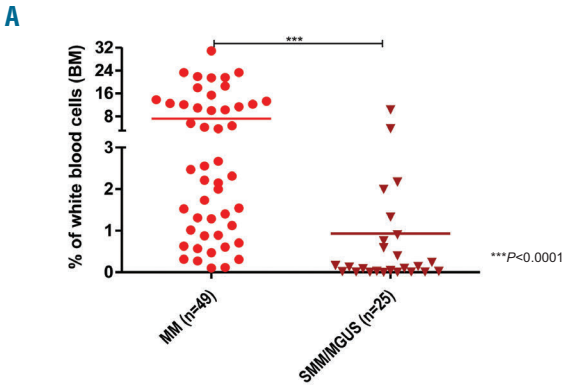
In conclusion, our study addressed the urgent need for adaptable and sensitive flow cytometry assays to evaluate treatment efficacy outside clinical trials as an alternative to other panels.[6] Our single-tube, eight-color MFC assay was practicable in all MM samples and high sensitivity was reached in 94% of patients' samples, demonstrating that routine measurement of patients, both in and outside clinical trials, undergoing routine BM puncture is possible, although high quality BM samples remain important.[20] This study demonstrated that the observed results regarding MRD negativity and improved PFS and OS are similar to those demonstrated in larger clinical trials and meta-analyses,[5,21] emphasizing the feasibility of the implementation of MRD determination in routine diagnostics.[2,11]

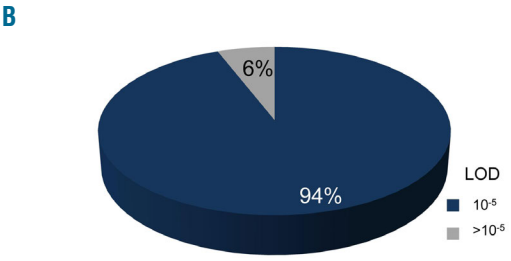
Strengths of this study include the fact that our thoroughly validated MFC single-tube assays minimize the laborious workflow without requiring special equipment[6] while preserving assay robustness and sensitivity. The importance of a readily available and standardized assay has been demonstrated by the EuroFlow[6] and MSKCC groups,[8,9] while the costs and software required, including database access, of the EuroFlow panel have been criticized[8,10] (*Online Supplementary Figure S1*).

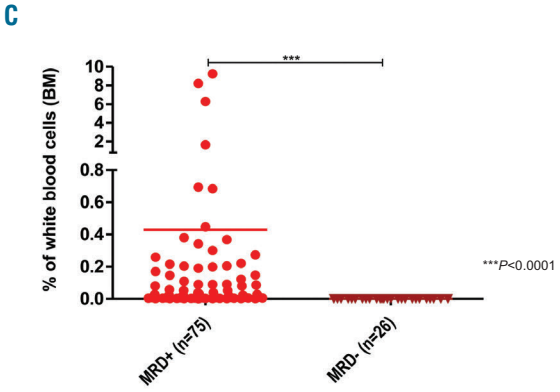
Table 2. The characteristics of patients with either minimal residual disease positive or negative bone marrow samples under treatment, not at initial diagnosis or disease progression, measured with the six- or eight-color panel.

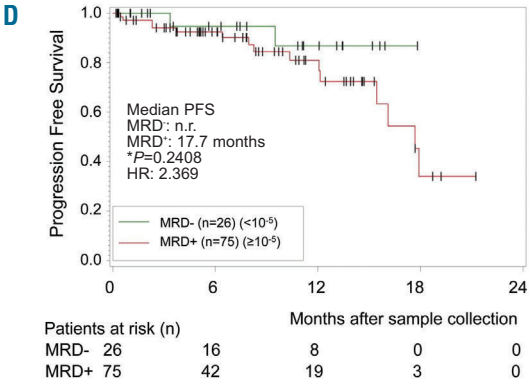
	Total cohort (n=91)[c]		MRD$^+$ (n=68)		MRD$^-$ (n=25)	
	N. (%)	Median (range)	N. (%)	Median (range)	N. (%)	Median (range)
Stage						
Post-CTx	10 (11)		9 (13)		1 (1)	
Post-ASCT/alloSCT	81 (89)		59 (87)		25 (99)	
Sex						
Female	41 (45)		30 (44)		12 (48)	
Male	50 (55)		38 (56)		13 (52)	
Age (years)						
ID		59 (28-84)		59 (32-84)		57 (28-74)
Sampling		62 (29-86)		63 (32-86)		59 (29-75)
Durie & Salmon (ID)						
I	10 (11)		9 (13)		4 (10)	
II	24 (26)		20 (29)		15 (38)	
III	57 (63)		39 (57)		21 (52)	
A/B	82 (90) / 9 (10)		63 (93) / 5 (7)		34 (85) / 6 (15)	
International Staging System (ID)						
I	35 (38)		24 (36)		12 (30)	
II	28 (31)		22 (32)		12 (30)	
III	28 (31)		22 (32)		16 (40)	
BM infiltration (%)						
Cytology		3 (0-28)		4 (0-28)		1 (0-7)
Pathology		5 (0-40)		5 (0-40)		0 (0-20)
Cytogenetics[a]						
Unfavorable	3 (3)		3 (4)		0 (0)	
Favorable	8 (9)		8 (12)		0 (0)	
Normal karyotype[d]	42 (46)		30 (44)		14 (56)	
Missing	38 (42)		27 (40)		11 (44)	
MM type						
IgG	70 (77)		52 (77)		19 (76)	
IgA	9 (10)		5 (7)		4 (16)	
Light chain only	12 (13)		11 (16)		2 (8)	
Light chain						
Kappa	67 (74)		53 (58)		16 (64)	
Lambda	24 (26)		15 (42)		9 (36)	
Last therapy before MRD determination						
Proteasome inhibitors	70 (77)		53 (78)		19 (76)	
Immunomodulatory drugs	6 (7)		4 (6)		1 (4)	
Antibodies	14 (15)		10 (15)		5 (20)	
Others	1 (1)		1 (1)		0 (0)	
Remission[b]						
Complete remission	18 (20)		16 (24)		3 (12)	
Very good partial remission	52 (57)		35 (52)		19 (76)	
Partial remission	18 (20)		14 (21)		3 (12)	
Stable disease	3 (3)		3 (3)		0 (0)	
MM progression						
Yes	15 (16)		14 (21)		1 (1)	
No	76 (84)		54 (79)		24 (99)	
Vital status						
Death	1 (1)		0 (0)		1 (4)	
Alive	90 (99)		68 (100)		25 (96)	

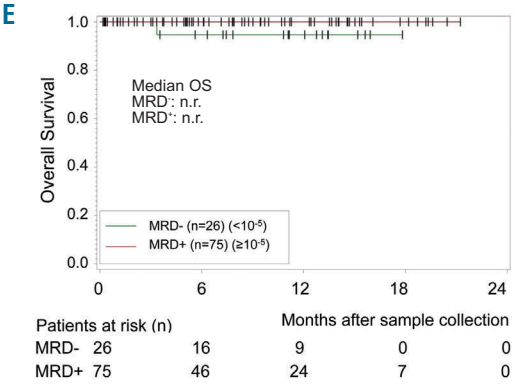
[a]Unfavorable defined as: +1q, t(4;14), t(14;16), del1p, cMYC, del17p; standard-risk defined as: hyperdiploidy, t(11;14), del13q, monosomy 13, del14q (IGH). [b]According to International Myeloma Working Group criteria. [c]Some patients were measured more than once during the period of sampling. Every patient was only counted once for each cohort. The number of both cohorts together is higher than that of the entire cohort because some patients were assessed sequentially. [d]Determined by fluorescence *in situ* hybridization analysis. MRD: minimal residual disease; post-CTx: after standard chemotherapy; post-ASCT: after autologous stem cell transplantation; post-alloSCT: after allogeneic stem cell transplantation; ID: initial diagnosis; BM: bone marrow.

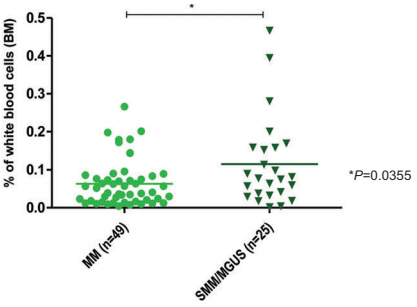

A
% of white blood cells (BM)
***
***P<0.0001
MM (n=49)
SMM/MGUS (n=25)

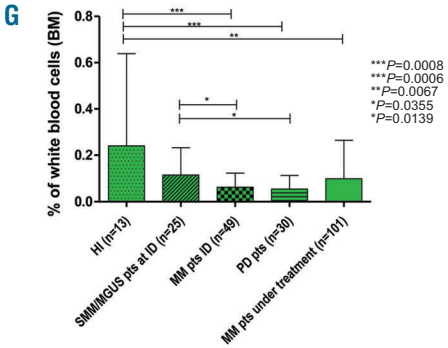

B
6%
94%
LOD
10-5
>10-5

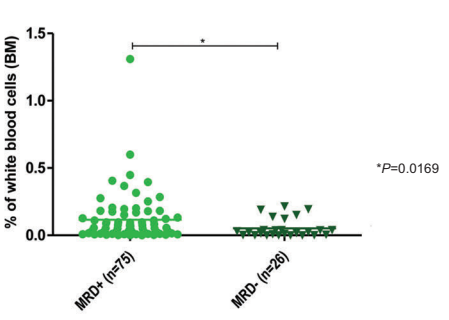

C
% of white blood cells (BM)
***
***P<0.0001
MRD+ (n=75)
MRD- (n=26)


D
Progression Free Survival
Median PFS
MRD-: n.r.
MRD+: 17.7 months
*P=0.2408
HR: 2.369
MRD- (n=26) (<10-5)
MRD+ (n=75) (≥10-5)
Months after sample collection
Patients at risk (n)
MRD- 26 16 8 0 0
MRD+ 75 42 19 3 0


E
Overall Survival
Median OS
MRD-: n.r.
MRD+: n.r.
MRD- (n=26) (<10-5)
MRD+ (n=75) (≥10-5)
Months after sample collection
Patients at risk (n)
MRD- 26 16 9 0 0
MRD+ 75 46 24 7 0


F
% of white blood cells (BM)
*
*P=0.0355
MM (n=49)
SMM/MGUS (n=25)


G
% of white blood cells (BM)
***P=0.0008
***P=0.0006
**P=0.0067
*P=0.0355
*P=0.0139
HI (n=13)
SMM/MGUS pts at ID (n=25)
MM pts ID (n=49)
PD pts (n=30)
MM pts under treatment (n=101)


H
% of white blood cells (BM)
*
*P=0.0169
MRD+ (n=75)
MRD- (n=26)

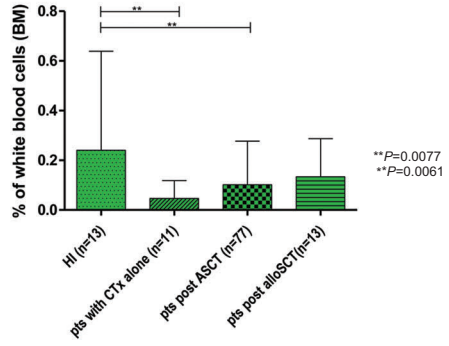

I
% of white blood cells (BM)
**P=0.0077
**P=0.0061
HI (n=13)
pts with CTx alone (n=11)
pts post ASCT (n=77)
pts post alloSCT(n=13)

Figure 2 (previous page). The level of aberrant and normal plasma cells in bone marrow samples and the influence of minimal residual disease detection on the outcome of patients. (A) Patients' samples at initial diagnosis were divided into two groups: those from patients with multiple myeloma (MM) in need of treatment and those from patients with smoldering multiple myeloma (SMM)/monoclonal gammopathy of undetermined significance (MGUS) not in need of treatment. As expected, the percentage of aberrant plasma cells (aPC) was significantly higher (***P<0.0001) in MM samples (n=49) than in SMM/MGUS samples (n=25). (B) In 94% of all minimal residual disease (MRD) samples (n=101) the limit of detection and a sensitivity of 10^{-5} could be reached. (C) Patients' samples after treatment were divided into two groups: MRD$^+$ (≥10^{-5}) and MRD$^-$ (<10^{-5}). The percentages of aPC in MRD$^+$ samples (n=75) were, expectedly, significantly higher (***P<0.0001) than those in MRD$^-$ samples (n=26). (D) Post-therapy bone marrow samples were divided into MRD$^+$ (≥0.001% aPC among total nucleated cells) and MRD$^-$ (<0.001% aPC among total nucleated cells) to estimate progression-free survival. The estimated median progression-free survival, determined using the Kaplan-Meier method, was 17.7 months for MRD$^+$ patients (n=75) and not reached for MRD$^-$ patients (n=26) (P=0.2408). The hazard ratio was 2.369 (95% confidence interval: 0.54-10.50). (E) Overall survival was determined for the MRD$^-$ (n=26) and MRD$^+$ (n=75) cohort. A big difference could not be observed because only one event was censored. [(F) Patients' samples at initial diagnosis were divided into those from patients with MM in need of treatment and those from patients with SMM/MGUS not in need of treatment. Interestingly, the percentages of normal plasma cells (nPC) were significantly higher (*P=0.0355) in SMM/MGUS patients than in MM patients. (G) Comparing nPC in healthy individuals (HI) and MM patients at different stages of the disease, the percentages of nPC in HI (n=13) were significantly higher (***P=0.0008) than those from MM patients at initial diagnosis (n=49), with progressive disease (n=30) (***P=0.0006) or under treatment (n=101) (**P=0.0067). Patients at initial diagnosis of SMM/MGUS (n=25) had significantly higher percentages of nPC compared to MM patients at initial diagnosis (*P=0.0355) or during progressive disease (*P=0.0139). No significant differences were observed between SMM/MGUS patients and HI or patients under treatment or among MM patients at initial diagnosis, during progression and under treatment. (H) Patients' samples after treatment were divided into MRD$^+$ (≥0.001% aPC among total nucleated cells) and MRD$^-$ (<0.001% aPC among total nucleated cells). The percentages of nPC were significantly higher (*P=0.0169) in MRD$^+$ samples than in MRD$^-$ samples. (I) Comparing nPC numbers in HI and patients treated with different modalities, the percentages of nPC were significantly higher in HI (n=13) than in patients after treatment with standard chemotherapy (n=11) (**P=0.0077) or after autologous stem cell transplantation (n=77) (**P=0.0061). No significant differences were observed between nPC numbers in HI and patients after allogeneic stem cell transplantation (n=13) or among the groups treated with the different modalities. The graphs show the mean (A, C, F, H) or mean ± standard deviation (G, I), Mann-Whitney U-test (A, C, F-I), and Kaplan-Meier method (D, E). BM: bone marrow; LOD: limit of detection; PFS: progression-free survival; n.r.: not reached; HR: hazard ratio; n: number; OS: overall survival; pts: patients; ID: initial diagnosis; PD: progressive disease; CTx: standard chemotherapy; ASCT: autologous stem cell transplantation; alloSCT: allogeneic stem cell transplantation.]

In follow-up studies, our numbers of patients could be increased and data analyzed after a longer follow-up. MRD samples at later time-points after intensive treatment regimens are of interest to determine whether higher MRD$^-$ rates can be achieved. Furthermore, imaging techniques (e.g., positron emission tomography) should be combined with MRD detection, since some patients show extramedullary MM progression without BM involvement.[22–24]

Limitations of the study include the relatively short time between treatment and MRD determination, due to routine BM puncture at day 30 after autologous SCT in 89% of our cohort and different treatment regimens; however, this reflects the routine BM evaluation schedule at our and other centers and increasing treatment diversity in MM. Furthermore, despite meticulous analysis of both MM cell lines and MM/SMM/MGUS patients' samples, both at initial diagnosis and after therapy, no head-to-head comparison with EuroFlow/MSKCC panels[6,8,9] was performed.

In summary, our results demonstrate a highly validated, straightforward approach for MRD determination in MM, which may represent a good alternative to others.[6] This MRD MFC assay can be used without special equipment and is easily implemented in routine clinical diagnostics. Furthermore, to the best of our knowledge, this is one of the first studies demonstrating that MRD detection is possible: results comparable to those of prior reports were obtained in >290 patients' samples routinely treated in and outside clinical trials, with a highly sensitive and resource-efficient, single-tube MFC assay.

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