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LETTER TO THE EDITOR

Circulating tumor cells as a biomarker for response to therapy in multiple myeloma patients treated within the GMMG-MM5 trial

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During the last 15 years, the outcome of patients with multiple myeloma (MM) has improved significantly as a result of therapy with novel drugs.[1] Up to 75–90% of fit patients reach CR or very good partial response according to the IMWG criteria.[2] Nevertheless, most of the patients suffer from relapse, indicating the presence of minimal residual disease (MRD).[2] Indeed, highly sensitive methods for detection of MRD, such as multicolor flow cytometry (MFC), allele-specific oligonucleotide PCR (ASO-PCR) and next-generation sequencing (NGS)-based assays, enable detection of residual tumor cells even in patients achieving clinical CR.[3–5] Presence of MRD in these patients is associated with a worse PFS and overall survival.[2–5] Recently, the IMWG has acknowledged these results in the new consensus criteria for response assessment in MM, which now includes MRD diagnostics when patients have reached CR and MRD negativity as the deepest response.[2] Along with the new consensus criteria, the IMWG pointed out that circulating tumor cells (CTCs) should be investigated for their value as a biomarker for response and prognosis since CTCs have been found in the PB of most patients at the time of diagnosis, and their level was identified as an independent prognostic factor.[2]

In this study, we performed a longitudinal quantitative analysis of CTCs and malignant plasma cells in the bone marrow (BM) in MM patients treated with novel agents and autologous stem cell transplantation (ASCT) using a highly sensitive ASO-PCR ($\leqslant 10^{-6}$). We aimed to examine if CTCs could be used as a minimal invasive biomarker for response to therapy beyond MRD diagnostics that are usually performed when patients reach CR. Samples were collected from patients who were treated within the open-label, randomized, multicenter phase III clinical trial MM5 for newly diagnosed MM patients of the German-speaking Myeloma Multicenter Group (GMMG, EudraCT no. 2010-019173-16),[6] and who reached CR or suspected CR until spring 2014 (Table 1; *N* = 41; 104 PB; 29 BM). BM samples were collected at diagnosis and at the time of CR or suspected CR (CR *N* = 18/29), and PB samples were collected at diagnosis and after the induction therapy (IT: PAd or VCD), ASCT and consolidation therapy (Cons.) (CR *N* = 33/104; Table 1). Additional 20 PB samples (at diagnoses and/or after IT, eight pairs) of 11 patients treated within the HOVON-65/GMMG-

Table 1. GMMG-MM5 and HOVON-65/GMMG-HD4 sample set

	N *Patients*	N *Samples—therapy regime per time point*										
		Diagnosis			*IT*			*ASCT*		*Cons.*		∑
		PAd	*VCD*	*PAD*	*PAd*	*VCD*	*PAD*	*PAd*	*VCD*	*PAd*	*VCD*	
GMMG-MM5 BM	23	0	0	—	2	3	—	8	8	4	4	29
GMMG-MM5 PB	41	11	15	—	11	10	—	13	16	15	13	104
GMMG-HD4 PB	11	—	—	10	—	—	10	—	—	—	—	20
GMMG-MM5 BM/PB pairs	18	—	—	—	2	3	—	5	8	2	4	24
	N Patients	*N* Samples—response to therapy per therapy regime										
		Diagnosis			PR		VGPR		CR			
		PAd	VCD		PAd	VCD	PAd	VCD	PAd	VCD		
GMMG-MM5 BM	23	—	—		2	1	3	5	9	9	29	
GMMG-MM5 PB	41	11	15		6	9	16	14	17	16	104	
GMMG-MM5 BM/PB pairs	18	—	—		2	1	2	5	5	9	24	
	N Patients	*N* Patients—clinical parameter at the time of diagnosis										
		PAd	VCD	PAD	HR		SR	ISS I	ISS II	ISS III		
GMMG-MM5 PB	41	20	21	—	16		25	16	15	10		
GMMG-HD4 PB	11	—	—	11	5		5	3	4	2		

Abbreviations: ASCT = autologous stem cell transplantation; BM = bone marrow; Cons. = consolidation therapy; HR = gain 1q21 more than three copies, deletion 17p13 and *t*(4:14); ISS = International Staging System; IT = induction therapy; PAd = bortezomib/doxorubicin/reduced dose dexamethasone (240 mL per cycle); PAD = bortezomib/doxorubicin/dexamethasone (480 mg per cycle); SR = low risk (all others); VCD = bortezomib/cyclophosphamide/dexamethasone; VGPR = very good partial response.

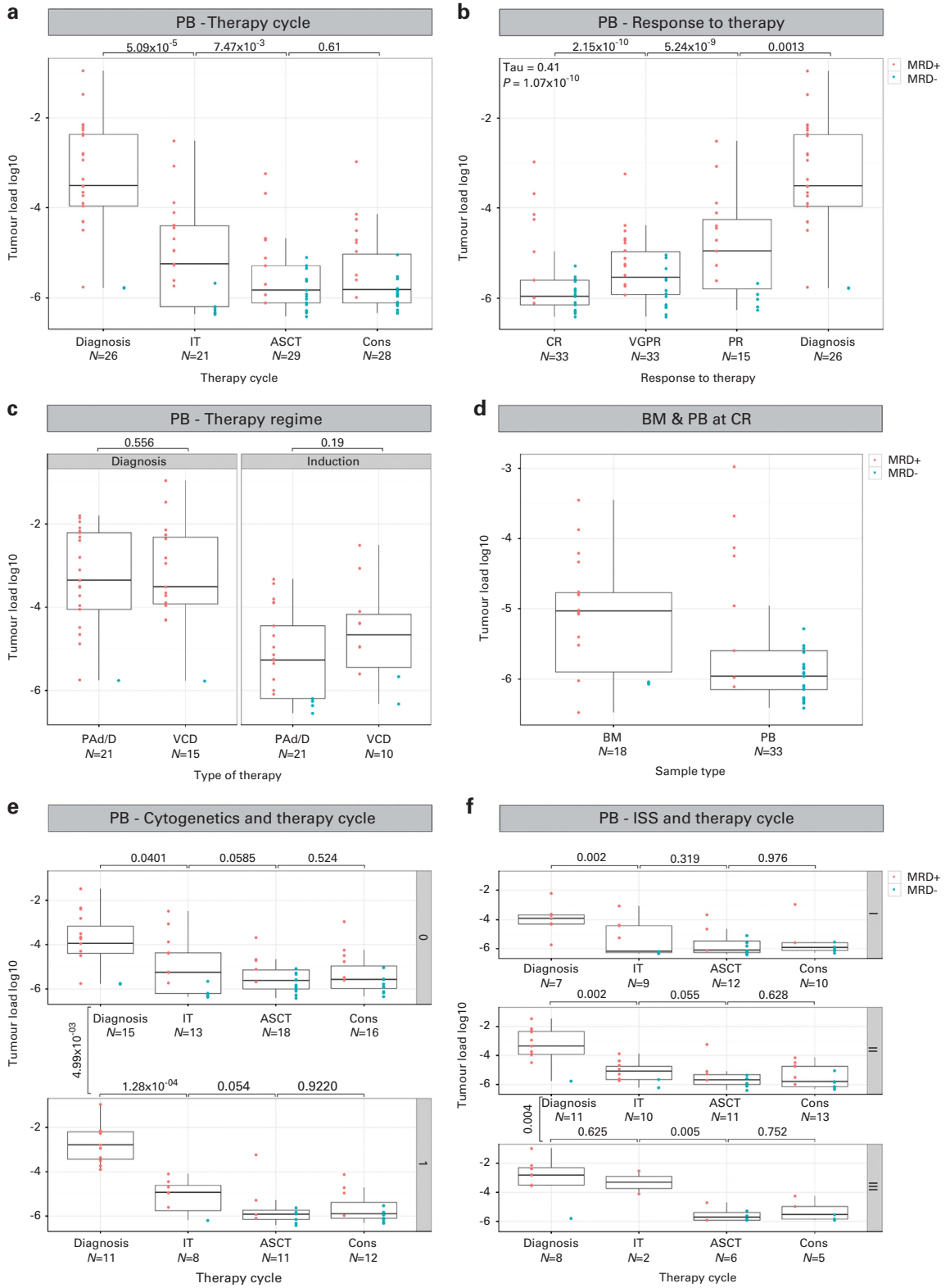

Figure 1. (**a**) GMMG-MM5—correlation between tumor load in PB and therapy cycle; $N = 106$. (**b**) GMMG-MM5—correlation between tumor load in PB and response to therapy. (**c**) GMMG-MM5 and GMMG-HD4—tumor load in PB at diagnosis and after different Bortezomib-based induction therapy regimes. PAd = bortezomib/doxorubicin/reduced dose dexamethasone (240 mL per cycle); VCD = bortezomib/cyclophosphamide/dexamethasone; PAD = bortezomib/doxorubicin/dexamethasone (480 mg per cycle). (**d**) GMMG-MM5—tumor load in BM and PB at the time point at which the patients had reached CR (after IT, after ASCT and after Cons.). (**e**) GMMG-MM5—correlation between tumor load in PB and therapy cycle, stratified for the presence or absence of high-risk cytogenetics (HR = amp(1q) more than three copies, deletion 17p13, *t*(4:14) and *t*(14:16); SR = low risk (all others)); HR = 1; SR = 0. (**f**) GMMG-MM5—correlation between tumor load in PB and therapy cycle, stratified for ISS Stage. Ordinary boxplots ignoring censoring. (**g**) GMMG-MM5—correlation between tumor load in BM and PB if PB is positive; $N = 14$ pairs. (**h**) Tumor load in BM and PB if PB is positive; $N = 14$ pairs.

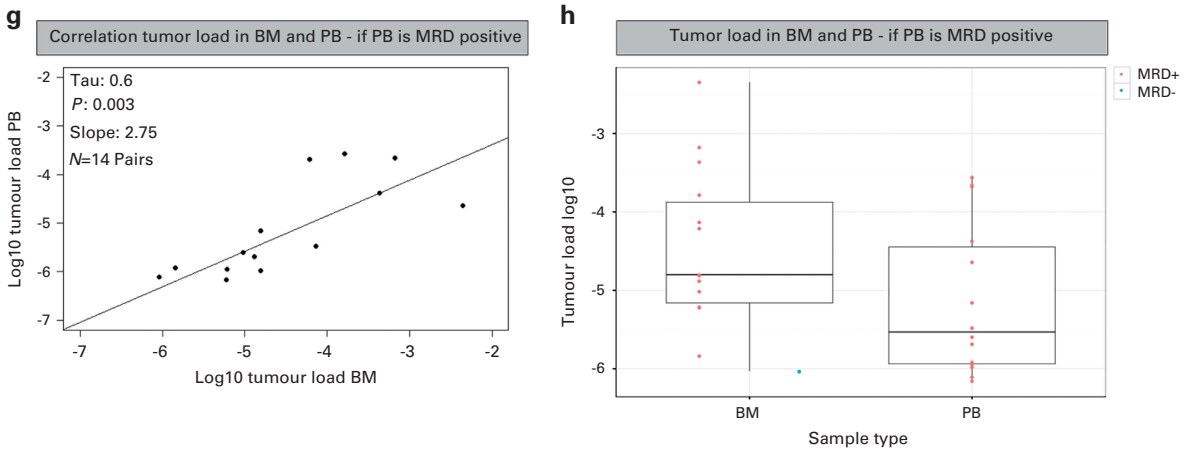

Figure 1. Continued.

HD4 Trial[7] were included to investigate differential impacts of the PAD/PAd regime (Table 1).

Tumor cell quantification was performed by patient-specific ASO-PCR assays designed to detect 1 tumor cell in 330 000 mono-nuclear cells (MNCs) in one PCR reaction and by extreme limiting dilution until no more amplification could be detected in at least 10 replicates.[8] The proportion of clonotypic cells in a sample was then calculated using the algorithm of 'extreme limiting dilution analysis'.[9] For MRD-negative results (MRD^-), a minimum of 10^6 cell equivalents had to be tested without any positive amplification if this amount of material was available to reach a sensitivity for $MRD^- < 1/10^6$.

Statistical analyses were carried out using the R package NADA[10] for left-censored data (Kendall's tau correlation coefficient, Akritas–Theil–Sen nonparametric line and Turnbull estimate of intercept). The Peto and Peto modification of the Gehan–Wilcoxon test was used for differences of the median tumor load.[10] MRD^- results were included as censored data, using the number of tested cells as individual censoring value for each sample. The median could not be calculated for groups that contained >50% censored data; in this case, mean values are presented. The data were analyzed for different risk strata according to International Staging System (ISS)[11] and cytogenetics at the time of diagnosis.[12,13] As high-risk (HR) cytogenetic markers, we included amp(1q) (more than three copies), deletion del (17p13) and the translocation *t*(4;14).[12,13] All other patients were defined as standard risk (SR).

Among all 104 measurements in PB samples, CTCs were detected in 54 (MRD^+; median 5.63×10^{-5}), with the lowest detectable number of CTCs of 7.75×10^{-7} (after ASCT, CR). The median sensitivity for samples without detectable CTCs (MRD^-) was 1.09×10^{-6} with the study wide weakest sensitivity of 9.08×10^{-6}.

At the time of diagnosis, CTCs were detected in 24 of 26 patients (92%; median relative load in MRD^+ 3.76×10^{-4}) with a maximum of 11% CTCs in PB MNCs. After IT, the number of CTCs was reduced significantly by 97% and reduced by an additional 86% after ASCT (Diagnosis-IT mean: 7.10×10^{-3} vs 2.07×10^{-4}, $P=5.09\times10^{-5}$; IT-ASCT mean: 2.07×10^{-4} vs 2.98×10^{-5}, $P=7.47\times10^{-3}$; Figure 1a; Supplementary Table 1). The most significant difference in CTCs was detected between the time of diagnosis and after ASCT (99.6% reduction; mean 7.1×10^{-3} vs 2.98×10^{-5}, $P=2.72\times10^{-9}$). Comparing the included Bortezomib-based ITs—PAd/PAD and VCD (Figure 1c), we could neither detect significant differences in the magnitude of CTC reduction from diagnosis to IT (98% vs 96%), nor in the number of CTCs after IT in the PAd/PAD- and VCD-treated patients (median VCD: 1.14×10^{-5}; median PAd/PAD: 1.83×10^{-6}; $P=0.191$; Supplementary Table 1).

Only 3 of 21 BM samples were MRD^- (14.3%) in patients in CR (median sensitivity of MRD^- 8.75×10^{-7}). The median relative tumor load in the MRD^+ patients was 1.56×10^{-5} (range 3.48×10^{-4} to 3.3×10^{-7}; Figure 1d; Supplementary Table 1).

With PB samples collected irrespective of response, we could show that CTCs were not only reduced significantly with every cycle of therapy, but that this reduction also positively correlated with clinical response (tau = 0.41; $P=1.07\times10^{-10}$; Figures 1a and b; Supplementary Table 1). Of note, in 8/19 patients in CR (42%), we detected CTCs (Figure 1d).

Stratifying the data for risk according to cytogenetics, we found a significantly higher number of CTCs at the time of diagnosis in HR patients than in SR patients (median: 1.6×10^{-3} vs 1.1×10^{-4}, $P=0.005$; Figure 1e). After IT, the number of CTCs was significantly reduced in HR patients (99.8% reduction) and SR patients (89% reduction) (HR median IT: 1.1×10^{-5}, $P=1.28\times10^{-4}$; SR median IT: 5.35×10^{-6}, $P=0.04$), and no significant difference after IT could be detected between the two risk groups ($P=0.95$) (Figure 1e; Supplementary Table 1).

Between the different ISS stages, no significant differences in the number of CTCs at the time of diagnosis and after ASCT were detected (Figure 1f; Supplementary Table 1). However, while patients with ISS I and II already showed a significant reduction of CTCs from diagnosis to IT (ISS I 88.9% reduction, $P=0.01$; and ISS II 99.5% reduction, $P=0.004$, respectively) in ISS III patients, CTCs were only reduced by ASCT (99.97% reduction, $P=0.005$) (Figure 1f; Supplementary Table 1).

Comparing the tumor load in BM and PB, we found that in only 3/24 pairs, were both entities MRD^- (12.5%; median sensitivity: BM 8.95×10^{-7}, PB 9.36×10^{-7}). In 16/24 pairs, BM was MRD^+, while PB was MRD^- (66.6%; median tumor load BM^+ 1.56×10^{-5}; median sensitivity PB^- 6.36×10^{-7}). In only 5/24 pairs, was PB MRD^+, but most interestingly, all but one corresponding BM sample was MRD^+. Adding an additional eight BM/PB pairs collected after stem cell mobilization or during maintenance therapy, we could confirm that as long as PB is MRD^+, BM is also MRD^+ ($N=14$ PB^+ pairs; Figure 1h; median BM^+ 6.3×10^{-5}; median PB^+ 6.9×10^{-6}; Supplementary Table 1). Further analysis showed a strong correlation between tumor load in PB and BM if the paired PB sample was MRD^+ (tau = 0.604; $P=$ 0.0031; Figure 1g). In the only PB^+/BM^- case, tumor load in PB was $7.75\text{x}10^{-7}$ and sensitivity of the BM measurement was 9.13×10^{-7}.

Taken together, our analysis showed both a significant correlation with the number of tumor cells in BM if PB was MRD^+ and a significant correlation of the number of CTCs with response to therapy. Accordingly, CTCs could as such be a promising minimal invasive biomarker for the general activity of the disease in the BM.

In comparison to other recently published studies about MRD diagnostics in BM at CR, our rates of MRD^- patients are low (14.3%).

This might be due to the fact that our MRD assay reaches a sensitivity that is even below 10^{-6}. When applying the so far best published sensitivity thresholds for MFC (10^{-5}) and NGS (10^{-6}) to our data for BM samples at CR, the numbers are well in line with published proportions of MRD^- patients with 42–68% MRD^- by MFC and 19–35% by NGS.[2–5] Nevertheless, by increased sensitivity, we were able to identify 43% more BM MRD^+ patients and 12% more PB MRD^+ patients at CR compared to MFC. This highlights the fact that sensitivity is essential for MRD diagnostics in BM as well as for the analysis of CTCs. We conclude that CTCs could serve as a surrogate for BM evaluations until PB is MRD^-, but cannot stand alone for MRD detection. Larger studies of CTCs in MM patients and the analysis of their effect on PFS and overall survival are needed to confirm and evaluate our findings. Future developments in improvement of MRD assay sensitivity and applicability, potential automatization, high-throughput applications and cost reduction will determine which assay serves best for the clinical application of MRD diagnostics and CTC evaluation.[14,15]

CONFLICT OF INTEREST

SH, NW, JN, MP, TH, MHu, UB, BH-D, MHä, JD, MG, HK, UG, MHo, PR, AJ, NP and KD declare no conflict of interest. MV and RA are employees of Janssen and hold stock in Johnson & Johnson; HJS—Celgene: honoraria, travel grants and Amgen: honoraria; KW—Honoraria and Advisory Board von Amgen, BMS, Celgene, Janssen, Novartis, Takeda; FL—advisory role: BioNTech, Bristol-Myers-Squibb, Eli Lilly, GANYMED Pharmaceuticals, Merck Sharp & Dohme, Roche Pharma AG. Lecture honoraria: Amgen, Astra Zeneca, Eli Lilly, Merck Sharp & Dohme, Roche Pharma AG, Servier. Research grant: Boehringer Ingelheim, Fresenius Biotech. Travel grants: Amgen, Bayer, Merck Sharp & Dohme, Roche Pharma AG, Taiho Pharmaceutical; IWB—scientific grants Jabssen-Cilag and Celgene. TM is an employee of inVentiv Health; PW—Honoraria and membership on Advisory Boards of Sanofi-Aventis. Membership on Advisory Boards and Travel Grants from Hexal AG; HG—research support (institutions): Celgene, Janssen, Chugai, Novartis, BMS; Advisory Boards (institutions): Janssen, Celgene, Novartis, Amgen Takeda, BMS; Honoraria: Celgene, Janssen, Novartis, Chugai, BMS.

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AUTHOR CONTRIBUTIONS

Conceived and designed the study: SH, NW and HG; Performed the experiments and analyzed the data: SH, NW, JN, MP, TH and MHu; Acquired study material and data: SH, NW, JN, MP, UB, BH-D, MHä, HJS, KW, JD, MG, HK, UG, FL, MHo, PR, IWB, AJ, TM, PW and HG; Interpreted the results: SH and NW; Drafted the manuscript: SH and NW; Revised the manuscript: SH, NW, JN, MP, TH, MHu, UB, BH-D, MV, RA, MHä, HJS, KW, JD, MG, HK, UG, FL, MHo, PR, IWB, ADH, AJ, KD, TM, PW and HG; Approved the final version: SH, NW, JN, MP, TH, MHu, UB, BH-D, MV, RA, MHä, HJS, KW, JD, MG, HK, UG, FL, MHo, PR, IWB, ADH, AJ, KD, TM, PW and HG.

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