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Supplemental Methods

Patient enrollment

All patients included in this prospective study (N=550) have attended the Department of Clinical Therapeutics of "Alexandra" Hospital (Athens, Greece) between January 2017 and March 2022 and were diagnosed with MM according to current diagnostic criteria. BM and matched PB samples were phenotypically analyzed in each patient using NGF cytometry as part of a routine diagnostic workout. Patients have been treated in a real-world setting and 62% of them were ineligible of receiving autologous stem cell transplantation (ASCT).

Next-generation flow cytometry

BM and PB samples were collected in EDTA-tubes and were processed with the proposed bulk lysis protocol. A minimum of 10 million cells were then stained for 30 min at 4^{o}C with the two 8-color panels that included the combinations: CD19-PECy7, CD27-BV510, CD38-FITC, CD45-PerCPCy5.5, CD56-PE, CD138-BV421 and CD117-APC and CD81-APCH7 for the surface tube or cytoplasmic kappa-APC and cytoplasmic lambda-APCH7 for the intracellular tube. Following the appropriate fixation and washing steps, a minimum of 6 million events were acquired per tube on a clinical BD FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA). The instrument set-up was performed according to the EuroFlow standard operating procedures (SOP) and cytometer performance was applied on a daily basis that allowed for the reliable reproducibility of the cytometric assessment.

Recorded samples were stored as FCS files and were then analyzed with the Infinicyt® software (Cytognos S.L., Salamanka, Spain). Plasma cells were selected based on their bright CD38 and CD138 expression, and myeloma cells were discriminated from their normal plasma cell counterpart due to their differential aberrant overexpression of CD56 and/or CD117 and/or aberrant underexpression of the rest of the surface markers. Clonality was also confirmed as a prerequisite for the discrimination of the myeloma cells in all samples. The NGF limit of detection (LOD) per sample was considered as the presence of at least 20 cells with the same aberrant features and light chain restriction per total number of nucleated cells (both tubes combined).

Mass cytometry

Cryopreserved BM cells were thawed in prewarmed RPMI supplemented with 10% fetal bovine serum (FBS), washed twice and then resuspended in fresh medium. Thawed cells were then treated with 100 U/ml DNAse for 20 min in phosphate-buffered saline (PBS), supplemented with 2 mM EDTA and washed with Maxpar cell staining buffer (CSB) followed by a blocking step (Human TruStain FcX, Biolegend). Cells were stained for cell surface markers with the MDIPA antibody panel according to the manufacturer's instructions followed by two washes with CSB and fixation (2% filtered formaldehyde solution, Sigma) for 20 min at room temperature. Finally, cells were stained in DNA intercalator solution (1:1000 dilution of 125 μM Cell-ID™ Intercalator-Ir) in Maxpar Fix and Perm buffer overnight (all from Standard BioTools). Prior to acquisition (performed the following day), cells were washed with CSB buffer and Cell Acquisition Solution (CAS) and resuspended with EQ Passport beads (1:10 dilution). Stained cell preparations were acquired with an acquisition rate at 100 to 300 events/s. Acquired data were normalized using Passport beads (Standard BioTools method) with CyTOF software (version 10.7.1014) and then were imported into Cytobank.

FlowSOM clustering were projected on the tSNE map using tSNE-CUDA with perplexity set to 50 and all other settings (iterations, learning rate and early exaggeration) set to default/automatic. Complementary analysis was performed on B cells upon manual gating on CD19+ cells and performing clustering/dimensionality reduction in Cytobank, using those markers related to B cell phenotype/functional status and selecting hierarchical consensus as the preferred clustering method.

Bone marrow immune profiling with multiparameter flow cytometry

Additional to the NGF panel, three 8-color panels were also used for the analysis of the bone marrow immune profiling in NDMM patients, two designed for the functional characterization of T cells and one panel for the characterization of the MDSC subsets and M1/M2 monocytes. The T cell panels included the markers: CD3-FITC, CD4-APC-Cy7, CD8-PerCPCy5.5, CD25-APC, FoxP3-PE, CD127-BV510, CD39-BV421, Ki67-BV510, CD45RA-PC7, CD45RO-PerCPCy5.5, CTLA4-BV421, and HLA-DR-

PC7 (BD Biosciences, San Jose, CA, USA; Biolegend, San Diego, CA, USA). The MDSC panel comprised the markers: CD14-FITC, CD11b-PE, 7-AAD, CD124-BV421, CD33-BV510, HLA-DR-PC7, CD15-APC, and lin (CD3, CD19, CD56)-APC-Cy7 (BD Biosciences). Both T cell panels were applied on whole lysed BM samples, whereas the MDSC panel was applied on mononuclear cells after Ficoll density gradient isolation.

Statistical analysis

In order to test for the normality distribution of values within each continuous variable group, we applied both D'Agostino & Pearson omnibus normality test and the modified Kolmogorov-Smirnov test. Based on this, statistical significant differences between groups were tested with the appropriate parametric (t-test for comparing 2 groups; one-way ANOVA for ≥ 3 groups) or non-parametric tests (Mann-Whitney for comparing 2 groups; Kruskal-Wallis for ≥ 3 groups). Differences in the mean fluorescent intensity (MFI) of the NGF markers between BM and matched PB samples were depicted on a relative change heatmap based on the formula $\frac{MFI(PB)-MFI(BM)}{MFI(PB)}$.

PFS was considered as the time period from diagnosis until disease progression or death from any cause, while overall survival (OS) was considered as the time from diagnosis until death from any cause. Time to MRD negativity was considered as the time from diagnosis to the first MRD negativity achievement assessed with NGF.

Supplemental Tables

Supplementary Table 1: **Immune subsets and their corresponding phenotypes analyzed for the bone marrow immune profiling**

Immune subset	**Markers' expression**
NGF MRD panel	
Plasma cells	$CD38^{++}CD138+$
B cells	CD19+CD45+
Naïve B cells	$CD19+CD27-CD38^{-/dim}CD45+SSC^{low}$
B cell precursors	$CD19+CD27-CD38^{++}CD45^{dim}SSC^{low}$
Memory B cells	$CD19+CD27+CD38^{-/dim}CD45+SSC^{low}$
T cells	$CD19-CD45+CD56-SSC^{low}$
CD27+ T cells	$CD19-CD45+CD56-CD27+SSC^{low}$
NK/NKT cells	$CD19-CD45+ CD56-SSC^{low}$
CD27+ NK/NKT cells	$CD19-CD45+CD56-CD27+SSC^{low}$
Neutrophils	$CD45^{dim}SSC^{high}$
Myeloid progenitors	$CD38+CD45^{dim}CD117+SSC^{high}$
Monocytes – TAMs	$CD38+CD45+CD81+SSC^{int}$
Mast cells	$CD45^{dim}CD117^{++}$
Erythroblasts	$CD38-CD45-SSC^{low}$
Erythroid progenitors	$CD38^{-/dim}CD45^{-/dim}CD117+SSC^{low}$
T cells panel	
T regulatory cells (Tregs)	$CD3+CD4+CD25+CD127^{low}FoxP3+$
Effector/effector memory Tregs (eff/eff mem Tregs)	$CD3+CD4+CD25+CD127^{low}FoxP3+CD45RA-CD45RO+HLA-DR-CTLA4+$
Terminal effector Tregs (teff Tregs)	$CD3+CD4+CD25+CD127^{low}FoxP3+CD45RA-CD45RO+HLA-DR+ CTLA4+$
Resting Tregs	$CD3+CD4+CD25+CD127^{low}FoxP3+CD45RA-CD45RO+HLA-DR-CTLA4-$
CD39+ suppressor Tregs (CD39 Tregs)	$CD3+CD4+CD25+CD127^{low}FoxP3+CD45RA-CD45RO+CD39+$
CD4+ T cells	CD3+CD4+
Naïve CD4+ T cells	CD3+CD4+CD45RA+CD45RO-
Effector/Effector memory CD4+T cells (eff/eff mem CD4+)	CD3+CD4+CD45RA-CD45RO+
CD8+ T cells	CD3+CD8+
CD8+ Tregs	CD3+CD8+CD25+FoxP3+
Memory CD8+ T cells	CD3+CD8+CD45RO+
HLA-DR regulatory CD8+ T cells (HLA-DR reg CD8+)	CD3+CD8+HLA-DR+
MDSCs panel	
Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs)	$CD14-CD11b+CD15+SSC^{high}$
Early myeloid-derived suppressor cells (eMDSCs)	Lin(CD3/CD14/CD15/CD19/CD56)-HLA-DR-CD33+
Monocytic myeloid-derived suppressor cells (M-MDSCs)	$CD11b-CD14+HLA-DR^{low/-}CD15-$
M1 monocytes	Lin(CD3/CD14/CD15/CD19/CD56)-CD14+CD124-
M2 monocytes	Lin(CD3/CD14/CD15/CD19/CD56)-CD14+CD124+

Abbreviations: int, intermediate expression; SSC, side scatter

Supplementary Table 2: **Comparison of clinical presentation features in patients with detectable or undetectable levels of CTCs**

Clinical parameters	Patients with undetectable CTCs N=57	Patients with detectable CTCs N= 493	P value
Age, years	67 (44-86)	68 (29 – 92)	ns
Male gender (%)	31/57 (54.4)	263/493 (53.3%)	ns
Hemoglobin, g/dL	12.2 (7.2-15)	10.3 (4 – 18)	< 0.001
Platelets, $x10^9$/L	254 (22-492)	237 (47 – 805)	ns
White blood cells, $x10^9$/L	6.6 (4.2-11.4)	6 (2.3 - 68)	ns
Neutrophils, /μL	3900 (1200-18000)	3800 (1100 – 21.700)	ns
Serum albumin, g/dL	4.1 (2.7-4.8)	3.8 (1 – 5.3)	0.02
Serum creatinine, mg/dL	0.85 (0.5-4.25)	0.93 (0.45 – 11.8)	ns
Serum Calcium, mg/dL	9.5 (6.5-14.5)	9.5 (6.3 – 15)	ns
β2-microglobulin, mg/L	2.8 (1.4-48.5)	3.7 (0.7 – 35)	0.02
LDH, U/L	170 (72-550)	175 (68 – 660)	ns
Bone marrow infiltration (%)	28 (5-80)	60 (6 – 100)	< 0.001
ISS stage			0.0002
I	30/57 (50.9%)	139/493 (28.2%)	
II	20/57 (35.1%)	189/493 (38.3%)	
III	7/57 (12.2%)	165/493 (33.4%)	
R-ISS stage			< 0.001
I	22/52 (42.3%)	105/443 (23.7%)	
II	26/52 (50%)	226/443 (51.1%)	
III	4/52 (7.7%)	118/443 (26.6%)	
Phenotypic classification			< 0.0001
MGUS like	17/57 (29.8%)	27/493 (5.5%)	
Intermediate	37/57 (64.9%)	238/493 (48.3%)	
MM-like	3/57 (5.2%)	228/493 (46.2%)	
High-risk cytogenetics*	8/52 (15.4%)	135/443 (30.4%)	0.02
M-protein			Ns
IgA	11/57 (19.3%)	106/493 (21.5%)	
IgG	33/57 (57.9%)	256/493 (51.9%)	
IgD	4/57 (7%)	12/493 (2.4%)	
Light chain only	6/57 (10.5%)	78/493 (15.8%)	
Non-secretory	3/57 (5.3%)	41/493 (8.3%)	
Kappa light chain	34/57 (59.6%)	291/493 (59%)	Ns

Numbers show percentages or median values with range in parenthesis
CTCs, Circulating tumor cells; ISS, International Staging System; LDH, lactate dehydrogenase
*High-risk cytogenetics is defined by the presence of t(4;14), t(14; 16) and/or del(17p13) and/or +1q

Supplementary Table 3: **Log-rank comparisons for PFS according to CTC log category. P values are shown in the lower left part of the table and hazard ratios with 95% CI in parenthesis in the upper right part of the table.**

CTC levels	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10^{-1}	X	n.s.	0.35 (0.14-0.89)	0.28 (0.10-0.76)	0.2 (0.07-0.62)	0.1 (0.02-0.43)
10^{-2}	n.s.	X	0.59 (0.34-1.02)	0.46 (0.25-0.84)	0.33 (0.17-0.64)	0.16 (0.08-0.35)
10^{-3}	<0.001	0.03	X	n.s.	0.56 (0.36-0.89)	0.28 (0.17- 0.46)
10^{-4}	< 0.0001	0.002	n.s.	X	n.s.	0.33 (0.19-0.58)
10^{-5}	< 0.001	< 0.001	0.02	n.s.	X	0.48 (0.26-0.92)
10^{-6}	< 0.0001	< 0.0001	< 0.001	< 0.001	0.03	X

PFS, Progression free survival; CTCs, circulating tumor cells; n.s, not significant

10^{-6} defines the range from undetectable CTCs to CTCs <0.0005% of total nucleated cells; 10^{-5} for CTCs ≥ 0.0005% to <0.005%; 10^{-4} for CTCs ≥ 0.005% to <0.05%; 10^{-3} for CTCs ≥ 0.05% to <0.5%; 10^{-2} for CTCs ≥ 0.5% to <5% and 10^{-1} for CTCs ≥ 5%.

Supplementary Table 4: **Log-rank comparisons for OS according to CTC log category. P values are shown in the lower left part of the table and hazard ratios with 95% CI in parenthesis in the upper right part of the table.**

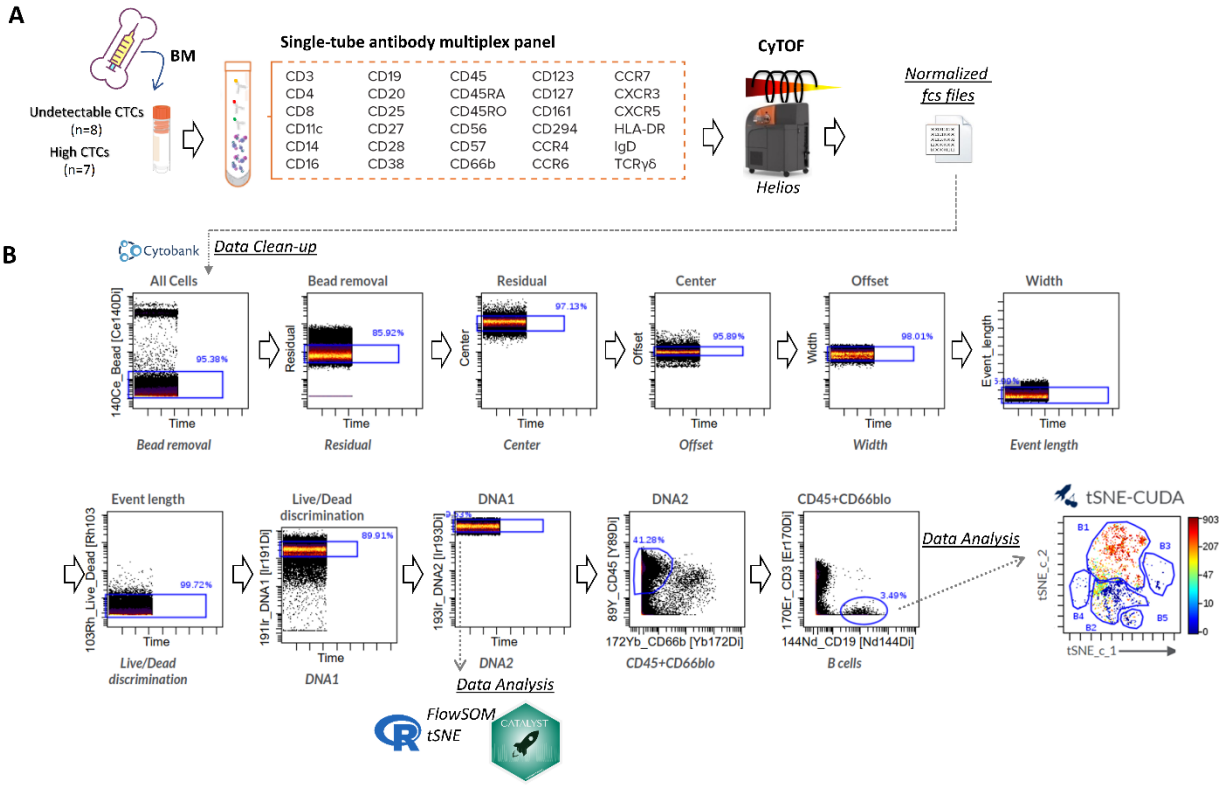
CTC levels	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10^{-1}	X	n.s	0.38 (0.09-1.5)	0.3 (0.07-1.3)	0.3 (0.07-1.37)	0.01 (0.001-0.08)
10^{-2}	n.s	X	n.s	0.38 (0.13-1.14)	0.39 (0.13-1.16)	0.06 (0.014-0.23)
10^{-3}	0.05	n.s	X	n.s	n.s	0.11 (0.037-0.34)
10^{-4}	0.01	0.03	n.s	X	n.s	0.11 (0.038-0.34)
10^{-5}	0.02	0.04	n.s	n.s	X	0.14 (0.04-0.47)
10^{-6}	< 0.0001	< 0.001	0.01	0.01	0.03	X

OS, Overall survival; CTCs, circulating tumor cells; n.s, not significant

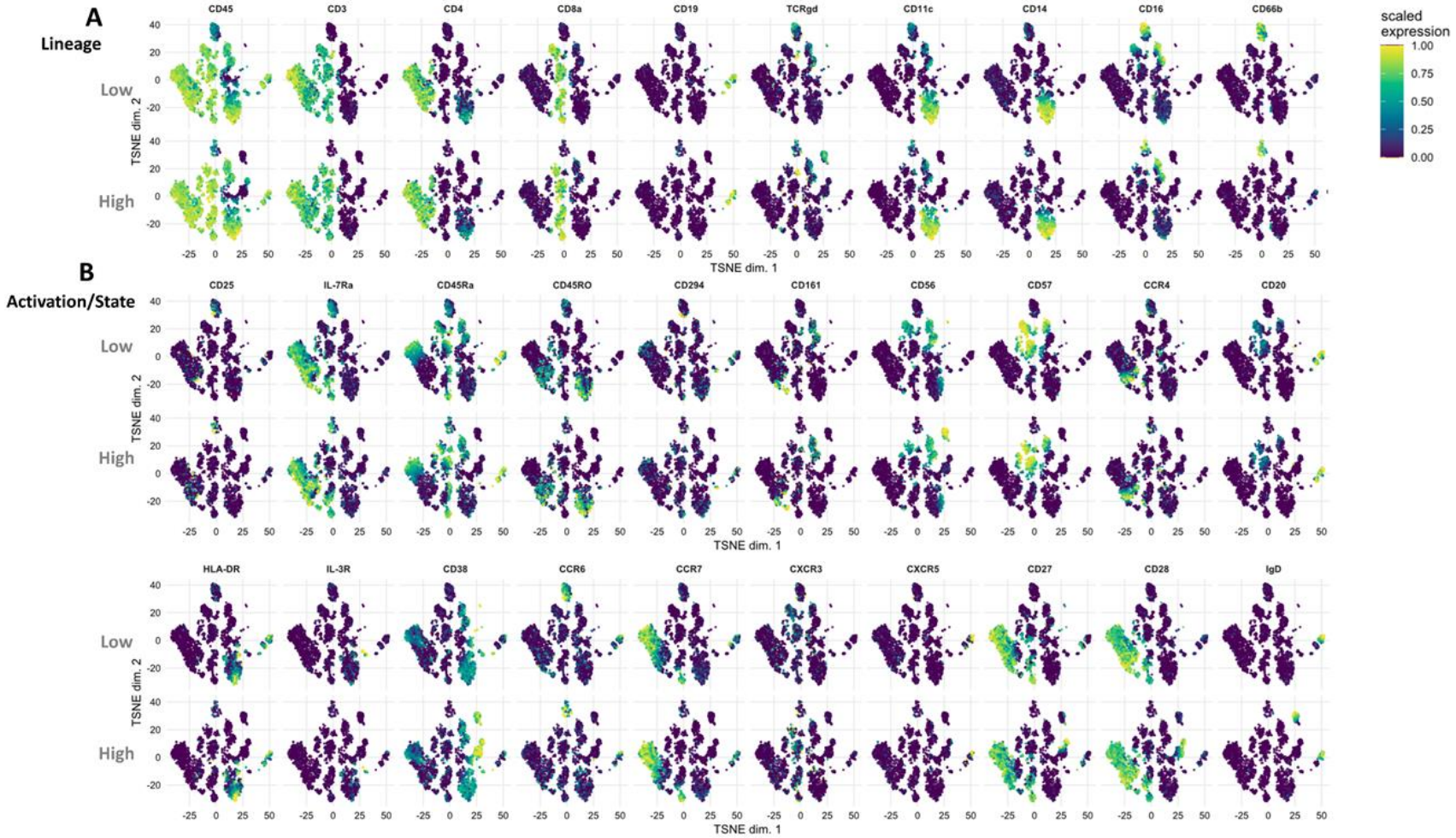
10^{-6} defines the range from undetectable CTCs to CTCs <0.0005% of total nucleated cells; 10^{-5} for CTCs ≥ 0.0005% to <0.005%; 10^{-4} for CTCs ≥ 0.005% to <0.05%; 10^{-3} for CTCs ≥ 0.05% to <0.5%; 10^{-2} for CTCs ≥ 0.5% to <5% and 10^{-1} for CTCs ≥ 5%.

Supplemental Figures

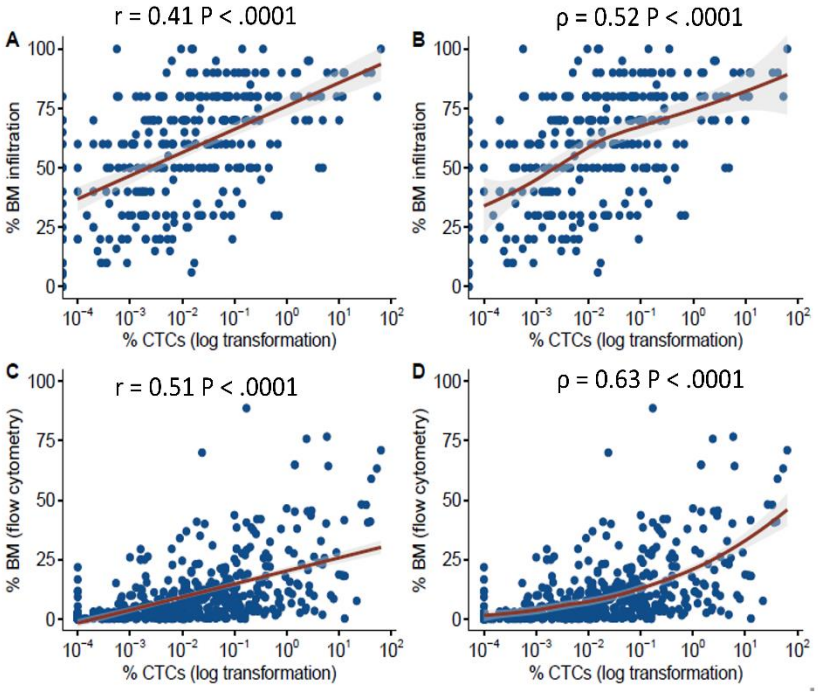
Supplementary Figure 1: **Experimental setup and workflow for high-dimensional cytometric analysis with CyTOF technology.** (A) Bone Marrow (BM) samples from patients with undetectable (n=8) and high (n=7) circulating tumor cells (CTCs) (>0.5%) were isolated and cryopreserved. After thawing, cells were stained with metal-tagged antibodies against the immune cell markers shown in the box (MDIPA, Standard BioTools). Labelled cells were acquired on a Helios mass cytometer and data produced (cytometry data, fcs format) were normalized and analysed using established analysis pipelines. (B) Data clean-up strategy included removal of cellular aggregates and ion cloud fusions using gating for 140Ce, 191/193Ir and Gaussian parameters center, width, offset and residual. Live singlet BM cells were exported for downstream analysis using the CATALYST pipeline or analysed in the Cytobank online platform.



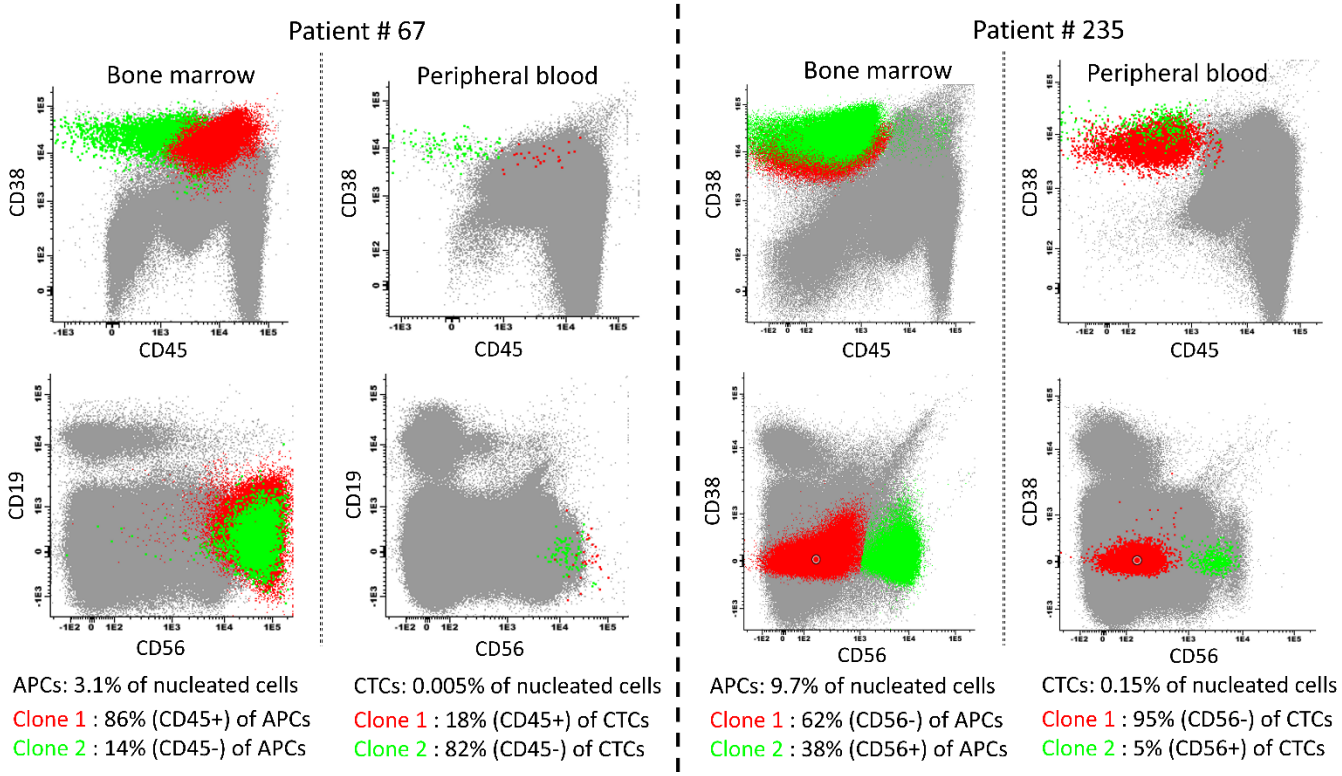
Supplementary Figure 2: **Representative protein expression patterns for the 30 markers of the MDIPA panel projected on the tSNE map.** (A) Markers for lineage selection. (B) Markers for the identification of activation state. Median scaled expression, blue - low expression to yellow – high expression.



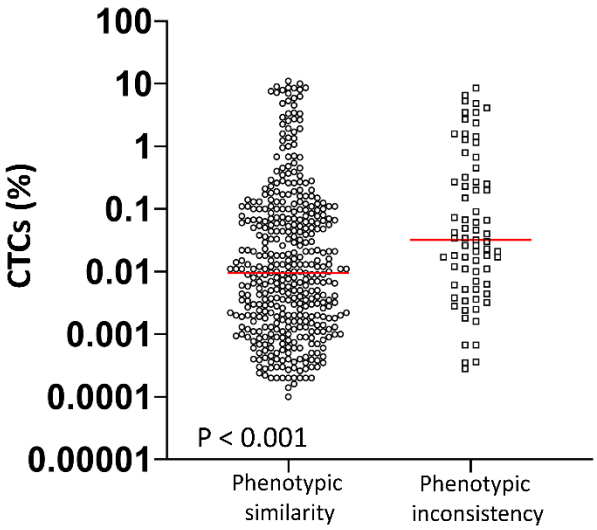
Supplementary Figure 3: **Correlations between the CTC levels and the BM infiltration.** The BM infiltration has been estimated morphologically (A,B) or with flow cytometry (C,D). A and C diagrams show correlations based on a linear regression model whereas B and D on a non-linear regression model. BM, bone marrow; CTCs, circulating tumor cells.



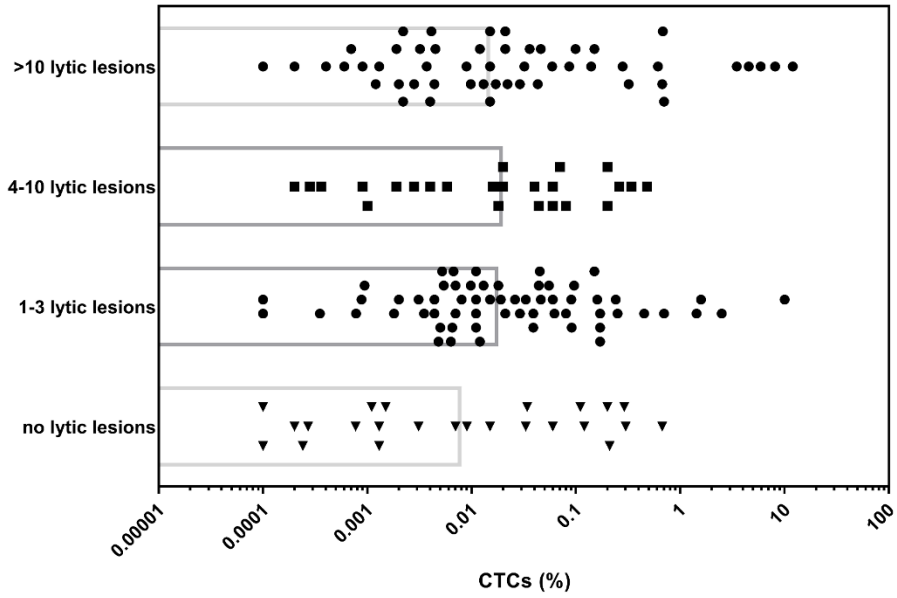
Supplementary Figure 4: **Representative cases showing phenotypic inconsistencies of clonal cells between bone marrow and peripheral blood.** The dot plots show all nucleated cells after debris exclusion. Clonal cells are shown in green and red colors (after selection in a CD38+CD138+ plot) and the remaining nucleated cells are shown in grey color. APCs, aberrant plasma cells; CTCs, circulating tumor cells.



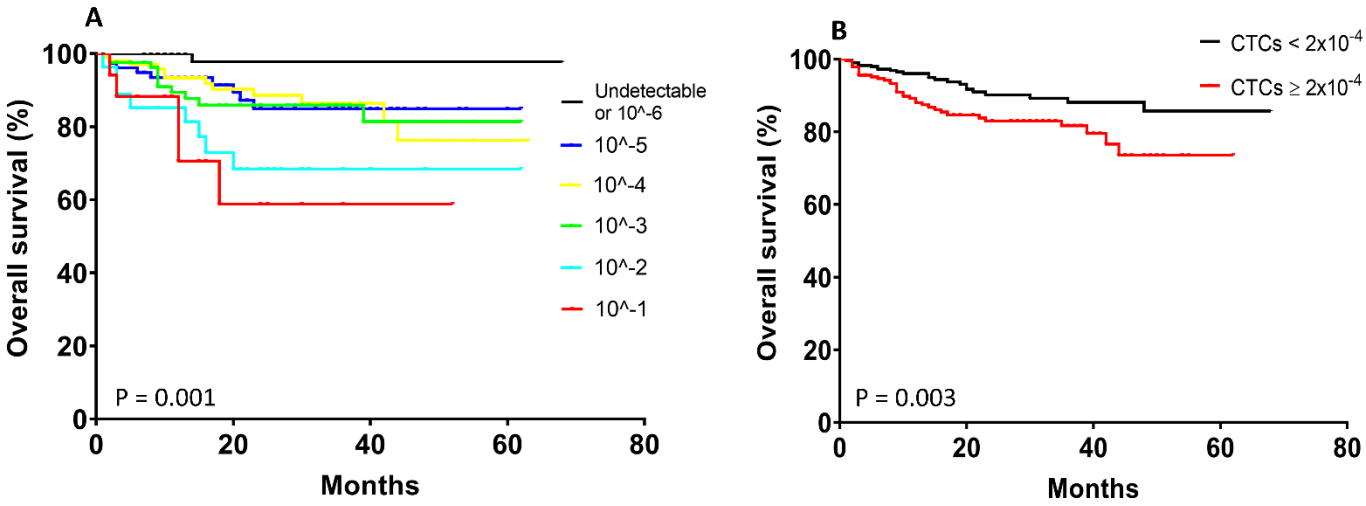
Supplementary Figure 5: **CTC distribution according to the presence of phenotypic inconsistency between bone marrow and peripheral blood.** Each dot corresponds to a single patient (n=420 patients with phenotypic similarity and n=73 patients with phenotypic inconsistency). Red lines show the median values in each category. CTCs, circulating tumor cells.



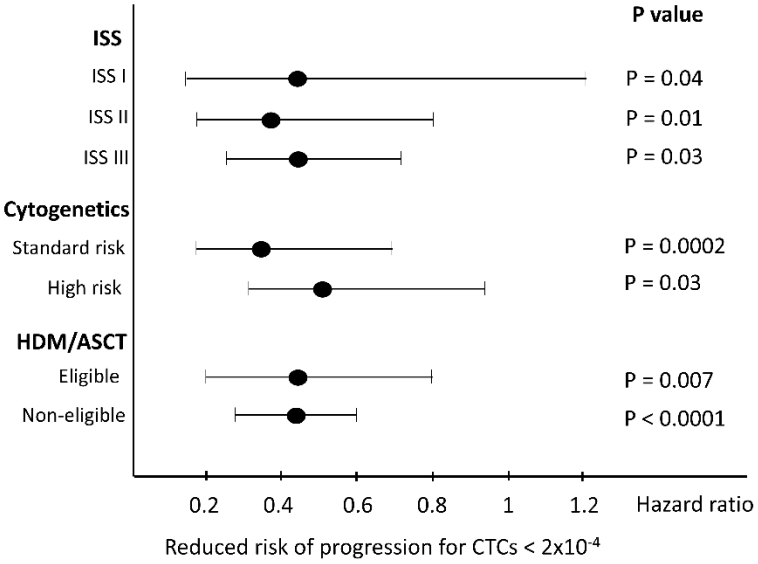
Supplementary Figure 6: **Association of circulating tumor cell (CTC) levels with the number of lytic lesions detected by whole-body computed tomography.** Each dot corresponds to a single patient (n=160 in total). The height of each bar depicts the median value of CTCs in each category.



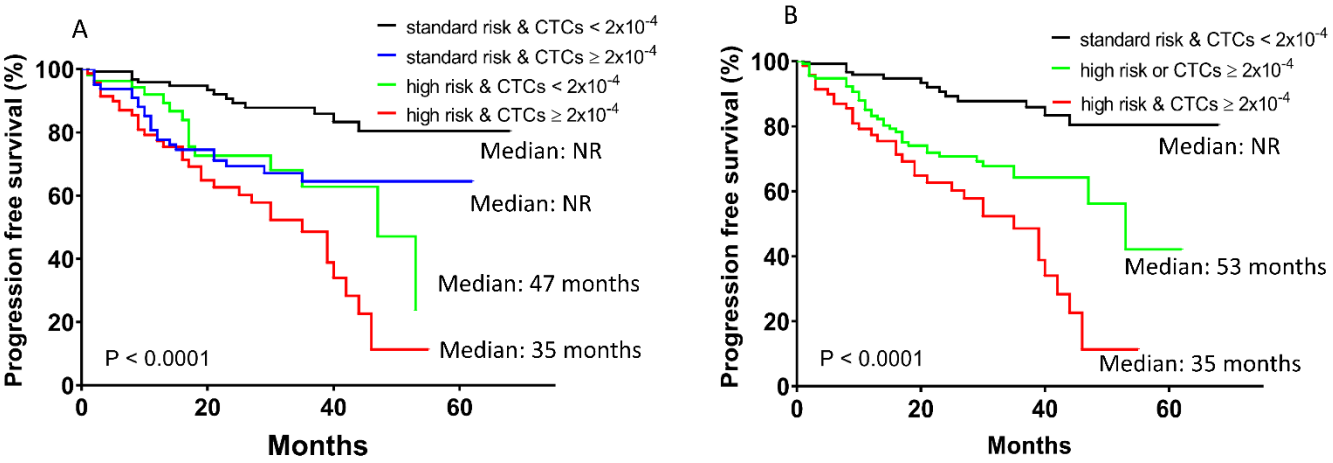
Supplementary Figure 7: **Overall survival (OS) according to the levels of circulating tumor cells (CTCs).** (A) OS per CTCs log increment. 10^{-6} stands for undetectable CTCs to CTCs <0.0005% of total nucleated cells; 10^{-5} for CTCs ≥0.0005% to <0.005%; 10^{-4} CTCs ≥0.005% to <0.05%; 10^{-3} for CTCs ≥0.05% to <0.5%; 10^{-2} for CTCs ≥0.5% to <5%; and 10^{-1} for CTCs ≥5%. (B) OS according to the clinically relevant cut-off point of CTCs defined at the level of $2x10^{-4}$.



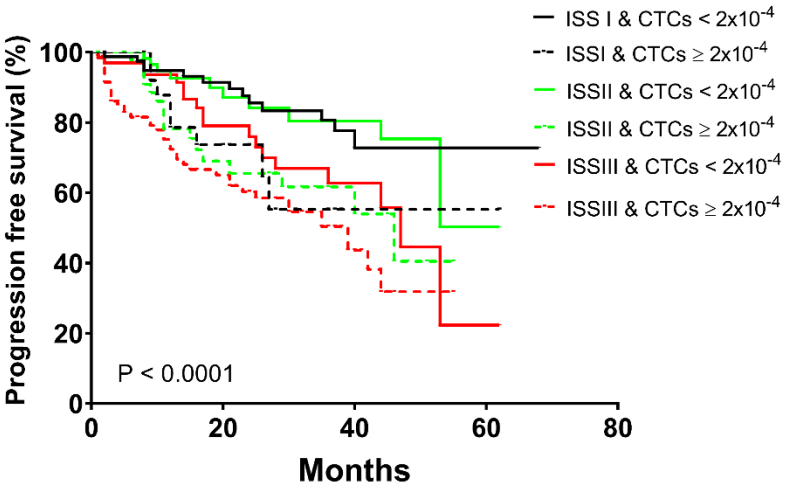
Supplementary Figure 8: **Subgroup analysis of prognostic parameters adjusted for the presence of circulating tumor cells (CTCs) below the clinical cut-off of 2x10-4.** HDM/ASCT, high-dose melphalan/autologous stem cell transplantation; ISS, International Staging System.



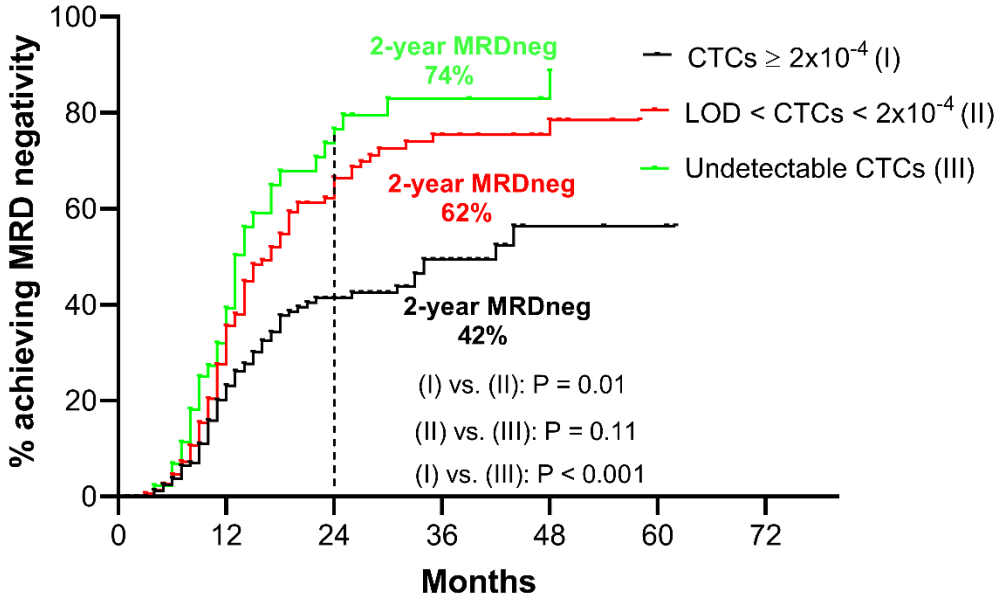
Supplementary Figure 9: **Progression free survival according to the cytogenetic status and the clinical CTC cut-off level defined at 2x10-4.** (A) Kaplan-Meier curves for all four resulting groups. (B) Kaplan-Meier curves after combining the two intermediate risk categories could better stratify patients in three distinct prognostic groups. High risk cytogenetics was defined by the presence of t(4;14), t(14;16) and/or del(17p13) and/or +1q. CTCs, circulating tumor cells; NR, not reached.



Supplementary Figure 10: **Progression free survival according to the ISS stage and the clinical CTC cut-off level defined at 2x10-4.** CTCs, circulating tumor cells; ISS, International Staging System.



Supplementary Figure 11: **Time to MRD negativity achievement according to the differential presence of CTCs at the time of diagnosis.** CTCs, circulating tumor cells; LOD, limit of detection; MRD, minimal residual disease



Supplementary Figure 12: **Frequencies of major immune subsets between patients with undetectable (n=8) and high CTCs (n=7) after metaclustering of the 26 populations obtained with FlowSOM analysis.** The frequency in each subset is calculated as (%) of the total bone marrow live singlets. DN, double negative; M, macrophages; NK, natural killer cells; TREG, regulatory T cells