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Running title: Baseline CTPCs and PFS in newly diagnosed multiple myeloma

Baseline circulating tumor plasma cells are associated with progression-free survival in newly diagnosed multiple myeloma: a single-center real-world NGF study

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Circulating tumor plasma cells (CTPCs) in peripheral blood provide a direct readout of myeloma dissemination. While CTPCs are increasingly recognized as prognostically relevant in newly diagnosed multiple myeloma (NDMM), practical cutoffs vary across cohorts and detection methods. Here, we evaluated baseline CTPCs quantified by next-generation flow cytometry (NGF) in a real-world NDMM cohort. Progression-free survival (PFS) was analyzed using the Kaplan-Meier method and compared across pragmatic CTPCs cutoffs (0.05% and 0.07%) and the cohort median (0.016%). CTPCs dichotomized at 0.05% significantly stratified PFS (log-rank p = 0.007), whereas 0.07% and the median cutoff yielded borderline separation. Patients with > 0.05% CTPCs at diagnosis showed a significantly shorter PFS compared to those with ≤ 0.05% CTPCs (median PFS of 436 vs. 1,227 days). Patients with > 0.05% CTPCs were at a significantly higher risk of event (progression or death) (hazard ratio HR 3.1, 95% confidence interval CI 1.3-7.3, p = 0.011). Peripheral blood normal plasma cells and bone marrow tumor plasma cells showed additional associations with PFS, while bone marrow normal plasma cells did not. R2-ISS stage and high-risk cytogenetics did not stratify PFS in this cohort. To conclude, baseline CTPCs measured by NGF identified a subgroup of NDMM patients at increased risk of progression in our real-world cohort. The cutoff of 0.05% should be considered pragmatic and assay-specific pending validation.

Key words: newly diagnosed multiple myeloma; circulating tumor plasma cells; next-generation flow cytometry; peripheral blood; prognosis; progression-free survival

Multiple myeloma (MM) remains clinically heterogeneous even in the era of modern therapy, and established prognostic factors fail to correctly predict outcomes in a significant proportion of patients [1, 2]. Baseline risk assessment in newly diagnosed MM (NDMM) is anchored in staging systems that integrate tumor burden surrogates and adverse biology, evolving from the International Staging System (ISS) to the Revised ISS (R-ISS) and more recently to the Second Revision of the ISS (R2-ISS) [3-5]. R2-ISS improves discrimination-particularly within intermediate-risk disease-

and is increasingly adopted as a baseline framework in routine NDMM practice [5]. Nevertheless, staging systems and binary cytogenetic grouping do not explicitly capture the biological dimension of dissemination, which may be central to early progression in a subset of patients [2, 5].

From a biological standpoint, an aggressive phenotype is reflected by the ability of malignant plasma cells to egress from the bone marrow and circulate in peripheral blood. Circulating tumor plasma cells (CTPCs) represent a direct cellular marker of systemic dissemination and can be quantified by flow cytometry at diagnosis [6-9]. Early and subsequent cohorts have consistently linked circulating plasma cells to adverse outcomes, supporting CTPCs as a clinically relevant baseline biomarker [6, 7, 10]. In a multicenter transplant-eligible NDMM cohort, Garcés et al. demonstrated that peripheral blood circulating tumor cell quantification by next-generation flow cytometry (NGF) carried substantial prognostic information and proposed a pragmatic cutoff (0.01%) for risk stratification [10]. In an independent transplant-eligible cohort, Bertamini et al. confirmed the adverse prognostic impact of higher CTPCs levels and reported an "optimal" cutoff near 0.07%, highlighting that practical thresholds may vary with cohort composition and methodology [11]. More recently, Kostopoulos et al. reported that a 0.02% CTPCs cutoff was independently associated with PFS and improved risk stratification when combined with conventional systems [12]. A recent systematic review and meta-analysis further support circulating plasma cells as a negative prognostic feature across multiple studies [13]. In parallel, a large pooled European analysis which included 2,364 NDMM patients and 68 patients with pPCL, confirmed CTPCs burden as an independent prognostic factor across clinical-trial and real-world cohorts [14]. The European CTC Consortium subsequently proposed a blood-based CTC staging system (CTC-SS), integrating CTC levels, ISS stage and LDH, based on 2,406 NDMM patients and 118 patients with pPCL; in that model, CTC burden is treated on a multilevel scale (< 0.002%, 0.002-0.02%, 0.02-0.2%, 0.2-2%, 2-5%, and > 5%) rather than as a single binary cutoff [15].

Frontline treatment in NDMM has changed rapidly, with daratumumab-based regimens increasingly used in both transplant-eligible and transplant-ineligible settings [16-19]. Importantly, the prognostic relevance of CTPCs has been reported under contemporary daratumumab-containing therapy, supporting the plausibility that CTPCs remain informative in current practice [20]. In this study, we evaluated whether baseline CTPCs quantified by NGF in peripheral blood are associated with PFS in a consecutive real-world NDMM cohort.

Patients and Methods

Patients. Fifty-five consecutive patients with symptomatic, treatment-indicated newly diagnosed multiple myeloma evaluated at our institution between 2021 and 2025 were included in the present

study. Baseline peripheral blood NGF assessment of circulating plasma cells was routinely requested as part of the diagnostic work-up during the study period. Patients were eligible if baseline PB NGF data obtained before initiation of first-line therapy and follow-up data allowing PFS assessment were available. Patients without available baseline PB NGF data, mainly because of logistical constraints related to sample acquisition or processing, were not included. Smoldering MM, relapsed MM and patients not indicated for treatment were not included. Patients were diagnosed according to the International Myeloma Working Group criteria for multiple myeloma [21]. The median follow-up time was 27.9 months. The data cut-off was 31 August 2025. Clinico-biological characteristics of patients are summarized in Table 1. The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of the Louis Pasteur University Hospital in Košice (protocol code 2021/EK/10064; approved on 21 December 2021). Written informed consent was obtained from all participants.

Next-generation flow cytometry analysis of circulating tumor plasma cells. Peripheral blood (PB) and bone marrow (BM) samples were collected into K3EDTA tubes, processed fresh within 24 h from collection, and handled using the same sample-preparation workflow. Preparation of peripheral blood (PB) and bone marrow (BM) samples for flow cytometric analysis was performed using the bulk lysis (BL) method according to EuroFlow SOP for bulk lysis in MRD panels Version 1.3. After determining the number of leukocytes in PB or BM, 2 ml of biological material was processed by the BL method. The final concentration of the isolated leukocytes in suspension was adjusted to 5×10^6-1×10^7 cells/100 µl in PBS/BSA/EDTA/NaN_3. The isolated leukocyte samples were labeled using a single-tube 10-color combination of monoclonal antibodies listed in Supplementary Table S1. Of note, Sato et al. showed strong correlation between a comparable single-tube 10-color combination and EuroFlow NGF for quantification of total and pathological plasma cells in BM MRD assessment [22]. After labeling of surface antigens, labeling of intracellular/cytoplasmic Ig light chains kappa (cyIgκ) and lambda (cyIgλ) was performed using the IntraPrep Permeabilization Reagent Kit (Beckman Coulter, USA) according to the manufacturer's recommendations. After labeling, samples were washed and resuspended in 500 µl of PBS/BSA/EDTA. Sample acquisition was performed using a Navios EX flow cytometer (Beckman Coulter, USA), aiming to acquire a minimum of 5×10^6 cellular events. Sample acquisition was stopped earlier when a sufficiently large cluster of cells (50 or more) was detected. Flow cytometer was set up and monitored using Flow-Set Pro and Flow-Check Pro fluorospheres using ClearLLab target values for the Navios EX flow cytometer (all Beckman Coulter, USA). The acquired data were analyzed using Infinicyt Version 2.0.6.b.009 software package (Cytognos, Spain). The immunophenotype of normal and pathological plasma cells and their relative % representation in

PB and BM, LOD ("limit of detection" defined as the identification of a minimum of 20 clonal myeloma cells in the total number of nucleated cells acquired) and LOQ ("limit of quantification" defined as a minimum of 50 clonal myeloma cells in the total number of nucleated cells acquired), median and mean values, and standard deviations were evaluated. CTPCs were expressed as the percentage of nucleated cells after exclusion of debris and doublets.

Statistical analysis. For statistical analyses, the SPSS software version 31.0.1.0 (IBM, USA) was used. To establish the statistical significance of differences observed between two or more groups, the non-parametric Mann-Whitney U and Kruskal-Wallis tests were used. For categorical variables, Fisher's exact test was used. For the analysis of correlations between two variables, Spearman's correlation coefficient was calculated. Progression-free survival (PFS), measured from diagnosis to first documented progression according to IMWG criteria or death from any cause, whichever occurred first, was estimated according to the Kaplan-Meier (KM) method and compared between groups by means of the log-rank test. Patients without progression or death were censored at the last available follow-up. Median follow-up time was estimated by reverse KM method. Hazard ratios (HRs) were estimated by univariate Cox proportional hazard models. Statistical significance was considered present once p-values were lower than 0.05.

Results

The levels of normal and tumor PCs in peripheral blood and bone marrow samples of 55 patients with symptomatic NDMM were analyzed by NGF. The number of total nucleated cells after exclusion of cell debris and doublets acquired per sample was 1,865,356±876,130 (227,119-4,191,128) (mean±standard deviation, range) for PB and 1,028,699±289,480 (80,817-1,418,234) for BM. Normal and tumor PCs were identified by their normal versus pathological immunophenotypic profiles and monotypic expression of cytoplasmic immunoglobulin light chains (Figures 1A, 1B). Both normal and tumor PCs were found in all PB and BM samples analyzed except one BM sample that was negative for the presence of normal PCs (Table 2). The median level of tumor PCs in PB (CTPCs) was 0.0159%. Twenty-five of 55 evaluable patients (45.5%) had CTPCs > 0.02%, 18/55 (32.7%) had CTPCs > 0.05%, 16/55 (29.1%) had CTPCs > 0.07%, 7/55 (12.7%) had CTPCs > 2%, and 4/55 (7.3%) had CTPCs > 5%. These high CTPCs values were retained as part of the real-world spectrum of symptomatic treatment-indicated NDMM and analyzed descriptively in the context of ultra-high CTPCs burden.

The levels of CTPCs displayed significant positive correlations with the percentage of bone marrow plasma cells by cytology (ρ=0.595, $p < 0.001$), M-protein levels (ρ=0.377, $p = 0.007$) and percentage of tumor PCs in the bone marrow (ρ=0.764, $p < 0.001$), as well as a significant inverse

correlation with normal PCs in BM (ρ=-0.309, p=0.023) (Table 3). The levels of CTPCs were significantly higher in NDMM patients with high-risk vs. standard-risk cytogenetics (2.35±7.90% vs. 0.56±2.35%, p=0.017), higher (III+IV) vs. lower (I+II) R2-ISS stage (2.67±8.02% vs. 0.03±0.05%, p=0.005), and patients who progressed or died vs. event-free patients (2.81±9.65% vs. 0.96±3.03%, p=0.021).

The median follow-up by reverse Kaplan-Meier analysis was 27.9 months (interquartile range 20.2-31.9 months). During follow-up, 21/55 patients (38.2%) experienced progression or death. PFS analyses according to five different CTPCs cutoffs are summarized in Table 4. Using the European CTC Consortium proposed 0.02% cutoff, patients with > 0.02% CTPCs had a shorter PFS than those with ≤ 0.02% CTPCs (median PFS 27.3 vs. 40.3 months; log-rank p=0.068; HR 2.23, 95% CI 0.92-5.42, p=0.075); however, this did not reach statistical significance and hence we were unable to confirm this threshold in our cohort. On the other hand, our exploratory dataset-derived optimal cutoff of CTPCs dichotomized at 0.05% significantly stratified PFS (log-rank p=0.007; Figures 2A-2D). Patients with >0.05% CTPCs at diagnosis showed a significantly shorter PFS compared to those with ≤ 0.05% CTPCs (median PFS of 14.3 vs. 40.3 months). Patients with > 0.05% CTPCs were at a significantly higher risk of event (progression or death) (hazard ratio HR 3.07, 95% confidence interval CI 1.29-7.31, p=0.011). Using a CTPCs cutoff of 0.07% yielded borderline separation (p=0.055) and dichotomization at the cohort median (0.0159%) also yielded borderline separation (p=0.053). Ultra-high cutoffs of 2% and 5% were not statistically significant in this small cohort, the > 5% subgroup including only four patients. Optimal cutoffs were also calculated for normal PCs in PB, tumor PCs in BM, and normal PCs in BM. Normal PCs in PB dichotomized at 0.02% stratified PFS (p=0.026), and tumor PCs in BM dichotomized at 1.69% stratified PFS (p=0.049), whereas normal PCs in BM dichotomized at 0.06% did not (p=0.903). Patients with > 0.02% normal PCs in PB at diagnosis showed a significantly shorter PFS than those with ≤ 0.02% (median PFS of 30.2 months vs. median PFS not reached NR). Patients with > 0.02% normal PCs in PB were at a significantly higher risk of progression/death (HR 4.6, 95% CI 1.1-19.8, p=0.042), but the very wide CI suggests small cohort size and few events. Patients with > 1.69% tumor PCs in BM at diagnosis showed a significantly shorter PFS than those with ≤ 1.69% (median PFS of 27.3 vs. 40.3 months). The percentage of normal PCs in BM did not significantly dichotomize PFS. The results of this analysis should be interpreted as exploratory, as the cutoffs were derived and tested on the same dataset. The R2-ISS stage (across four groups) did not significantly stratify PFS (p=0.190), and binary high- vs. standard-risk cytogenetics likewise showed no stratification of PFS (p=0.964). Complete response (CR) at the data cut-off strongly separated PFS (p=0.003), with substantially fewer PFS events among patients who achieved CR. Patients who achieved CR

showed a significantly longer PFS than those with a worse response to therapy (median PFS NR vs. 21.9 months).

Treatment distribution is summarized in Supplementary Tables S2 and S3. Twenty-five patients (45.5%) received daratumumab-based first-line therapy and 27 patients (49.1%) underwent autologous stem cell transplantation. PFS did not significantly differ according to daratumumab use alone (log-rank p=0.262) or ASCT performed vs. not performed (log-rank p=0.998). Daratumumab-based therapy was not significantly imbalanced between the ≤ 0.02% and > 0.02% CTPCs groups (16/30, 53.3% vs. 9/25, 36.0%; Fisher p=0.278), and ASCT was likewise balanced (14/30, 46.7% vs. 13/25, 52.0%; Fisher p=0.789) (Supplementary Table S2). In subgroup analyses, the CTPCs signal was more apparent in patients treated without daratumumab: at the 0.02% cutoff, > 0.02% CTPCs were associated with shorter PFS in the non-daratumumab subgroup (log-rank p=0.034; HR 3.02, 95% CI 1.04-8.80, p=0.043), whereas no separation was observed in the daratumumab-treated subgroup (log-rank p=0.948; HR 0.94, 95% CI 0.16–5.69, p=0.948). The same variables stratified by our exploratory dataset-derived optimal 0.05% CTPCs cutoff are presented in Supplementary Table S3. Daratumumab-based therapy was not significantly imbalanced between the ≤ 0.05% and > 0.05% CTPCs groups (20/37, 54.1% vs. 5/18, 27.8%; Fisher p=0.087), and ASCT was likewise balanced (19/37, 51.4% vs. 8/18, 44.4%; Fisher p=0.775). In subgroup analyses using the 0.05% CTPC threshold, the adverse prognostic effect of higher CTPCs was retained in patients not treated with daratumumab in the first line (median PFS 14.3 vs. 40.3 months, log-rank p=0.016; Cox HR 3.26, 95% CI 1.18-9.02, p=0.023). In daratumumab-treated patients, the direction of effect was similar but not statistically significant (median PFS NR vs. NR, log-rank p=0.359; Cox HR 2.26, 95% CI 0.38-13.60, p=0.372), most likely because of the very low number of PFS events in this subgroup. These subgroup analyses should be interpreted cautiously because the daratumumab subgroup included only five PFS events.

Discussion

The principal finding of our study is that baseline circulating tumor plasma cells (CTPCs) quantification in peripheral blood by next-generation flow cytometry (NGF) identified a subgroup of newly diagnosed multiple myeloma (NDMM) patients with significantly shorter progression-free survival (PFS), with the clearest separation observed at an optimal cutoff of 0.05%. By contrast, nearby cutoffs such as 0.02%, 0.07% and the cohort median (0.0159%) produced only borderline discrimination. This pattern supports the concept that CTPCs burden is prognostically relevant, while also suggesting that practical cutoffs are assay-specific and cohort-specific rather than

biologically universal [10-15, 20, 23]. Since our optimal cutoff of 0.05% is derived from a small dataset of 55 patients, it is not proposed as a new clinical cutoff.

Our results are consistent with a substantial body of evidence showing that circulating plasma cells reflect aggressive myeloma biology. Earlier flow cytometric studies demonstrated that circulating plasma cells are associated with advanced disease features and inferior survival in NDMM [6, 7]. More recent NGF-based work has shown that CTPCs are detectable in virtually all active MM cases and that increasing CTPCs burden is associated with progressively worse outcome [10-13, 23]. In transplant-eligible NDMM, Garcés et al. demonstrated that CTPCs could be used for noninvasive risk-stratification of MM patients based on their numbers and genetic profile [10], whereas Bertamini et al. showed that high CTPCs levels represent an easy-to-assess, robust, and independent high-risk factor and reported an optimal CTPCs cutoff of 0.07% [11]. Kostopoulos et al. subsequently provided evidence supporting a 0.02% threshold in a cohort including both transplant-eligible and transplant-ineligible patients [12]. In addition, a recent meta-analysis supports the adverse impact of elevated CTPCs on both PFS and OS across heterogeneous cohorts and detection methods (immunofluorescence, conventional morphology, polymerase chain reaction, multi-parameter flow cytometry, next-generation flow cytometry) [13]. The large European pooled analysis presented at EHA2025 and IMS2025 further confirmed the independent prognostic value of CTPCs burden in a broad dataset combining trial and real-world cohorts [14]. The subsequent EMN2026 abstract presenting the results of the European CTC Consortium moved the field further by proposing a multilevel blood-based staging system (CTC-SS) incorporating CTPCs levels, ISS and LDH rather than relying on a single binary cutoff [15]. Within this context, the 0.05% cutoff identified in our series should be interpreted as a pragmatic cutoff that performed best in this dataset rather than as an absolute biological boundary.

In our analysis, the 0.02% cutoff proposed by European CTC Consortium showed a numerical but non-significant PFS separation, whereas our optimal 0.05% cutoff showed the strongest separation and the 0.0159% (cohort median) and 0.07% thresholds showed borderline separation. Therefore, our data should not be interpreted as statistical validation of the 0.02% threshold or as evidence for establishing an alternative cutoff. This pattern is not unexpected in a cohort of 55 patients with 21 PFS events and heterogeneous treatment exposure. Despite obvious limitations, single-center cohorts may still be valuable for testing whether the general CTPCs signal is reproduced in real-world practice. From this perspective, the concordant direction of effect across the 0.0159%, 0.02%, 0.05% and 0.07% cutoffs are supportive, but larger cohorts would be required for formal threshold validation and integration into risk models.

The biological plausibility of CTPCs as a prognostic biomarker is strong. The presence of clonal plasma cells in circulation is unlikely to represent only a passive spillover phenomenon from the marrow. Rather, it probably reflects acquisition of dissemination-competent features by the malignant clone. Studies integrating NGF with genomic and transcriptomic analyses have shown that circulating tumor cells capture relevant molecular information, may derive from anatomically distinct marrow or extramedullary sites, and display transcriptional programs linked to trafficking, proliferation, and disease dissemination [24, 25]. More recently, Garcés et al. showed that elevated CTPCs levels reflect high proliferation and genomic complexity in MM, further supporting CTPCs as a marker of adverse disease biology rather than only tumor burden [26]. Thus, CTPCs may integrate several adverse dimensions simultaneously, including tumor burden, spatial heterogeneity, and the ability of the clone to escape the marrow microenvironment. This may help explain why a peripheral blood biomarker can remain informative even when conventional staging variables show limited discriminatory power in a modest-sized real-world cohort.

Another important consideration is the treatment context. Our cohort included both transplant-eligible and transplant-ineligible patients, and a substantial proportion received daratumumab-based first-line therapy, which more closely reflects contemporary routine practice than older single-regimen series. Phase 3 studies have established the benefit of daratumumab-containing combinations across NDMM settings, including CASSIOPEIA and GRIFFIN in transplant-eligible disease and MAIA and ALCYONE in transplant-ineligible disease [16-19]. Importantly, the adverse prognostic impact of CTPCs has also been confirmed in patients treated with daratumumab, bortezomib, lenalidomide, and dexamethasone, indicating that CTPCs remain clinically informative despite increasingly effective frontline therapy [20]. In our subgroup analyses, the CTPCs-associated PFS signal was more apparent in patients treated without daratumumab, whereas the daratumumab-treated subgroup included only five PFS events and cannot be used to infer treatment-specific interaction. Therefore, our findings support the view that CTPCs are not merely a biomarker from the pre-daratumumab era, but remain relevant in current NDMM management.

The four patients in our cohort with CTPCs values above 5% merit specific comment. These patients were retained because they were part of the consecutive symptomatic treatment-indicated NDMM cohort. Nevertheless, very high levels of circulating clonal plasma cells almost certainly define an ultra-high-risk biological subset [15, 27-29]. The International Myeloma Working Group lowered the morphologic threshold for pPCL to ≥ 5% circulating plasma cells in peripheral blood smears, and subsequent work has shown that even lower levels detected by flow cytometry-particularly above 2%-identify a plasma cell leukemia-like form of MM with very poor outcome [27, 28]. The Czech analysis by Jelinek et al. demonstrated that ≥ 2% CTPCs by flow cytometry

identify a plasma-cell-leukemia-like MM subset with poor outcome [28], while Bezdekova et al. further emphasized the relevance of flow-cytometric assessment of circulating plasma cells in primary and secondary plasma cell leukemia [29]. Thus, retaining these patients in the analysis is justifiable from a real-world standpoint, but their presence should be acknowledged as a potential contributor to the observed prognostic signal.

The correlation analyses in our series further reinforce the biological relevance of CTPCs. Higher CTPCs levels were associated with greater marrow plasma cell infiltration, higher M-protein levels, and higher bone marrow tumor plasma cell percentages, which is what would be expected from a dissemination marker linked to tumor burden. At the same time, CTPCs may outperform marrow-based measurements in some patients because bone marrow assessment is inherently vulnerable to hemodilution, patchy infiltration, and spatial sampling error [30, 31]. Multiple myeloma is a spatially heterogeneous disease, and a single iliac crest aspirate may incompletely represent whole-body disease distribution [32, 33]. From this perspective, peripheral blood CTPCs quantification may provide complementary information rather than simply duplicating what is already captured by bone marrow examination.

We also observed that bone marrow tumor plasma cells stratified PFS, whereas bone marrow normal plasma cells did not. This is not unexpected, since tumor plasma cell content is directly linked to disease burden, while normal plasma cell representation may be influenced by broader marrow ecology, treatment exposure, and sample quality. The association between peripheral blood normal plasma cells and PFS in our cohort is more difficult to interpret biologically. Because normal plasma cells in blood are not an established high-risk biomarker in NDMM, this finding should be regarded as exploratory and hypothesis-generating.

An additional finding was the lack of significant PFS discrimination by R2-ISS and by binary high-risk cytogenetics. This should not be interpreted as evidence against the clinical validity of either parameter. R2-ISS is a robust prognostic system derived from large datasets and subsequently validated in several real-world cohorts [5, 34-36]. However, these models were developed in substantially larger populations than ours, and their performance may be attenuated in a modest-sized single-center series with heterogeneous treatment exposure and a limited number of events. More specifically, this analysis had limited statistical power because only 55 patients and 21 PFS events were available, the R2-ISS groups were unevenly distributed, and the smallest categories contained very few patients (R2-ISS I, n=6; R2-ISS IV, n=4). In addition, R2-ISS was primarily developed for overall survival, whereas our endpoint was PFS in a heterogeneous real-world cohort with variable frontline therapy, daratumumab exposure, and ASCT use. Conversely, several studies suggest that circulating plasma cells can refine or complement standard staging systems, including

R-ISS/R2-ISS-based approaches [37, 38]. Our data fit well with that concept: in this cohort, CTPCs appeared to capture clinically relevant risk information that was not fully reflected by baseline staging alone.

Complete response at the data cutoff also showed strong separation of PFS, but because response is a post-baseline variable, treating it as a baseline prognostic factor would risk immortal time bias; in the present manuscript it is therefore better interpreted as a descriptive post-treatment correlate of outcome rather than as a pretreatment predictor.

Our present study has several limitations. It is single-center and based on a relatively small cohort, which limits the precision of cutoff selection and reduces power for more granular subgroup analyses. In addition, the proposed 0.05% cutoff was derived within the same dataset in which it was tested and should therefore be considered exploratory. Treatment was heterogeneous, reflecting real-world practice but complicating causal interpretation, and subgroup analyses according to daratumumab exposure and ASCT performed were exploratory. On the other hand, the study also has important strengths: all patients were evaluated using a sensitive NGF-based approach, the cohort reflects real-world contemporary NDMM practice, and the observed CTPCs signal was coherent with established biological and clinical knowledge. Overall, our results support baseline CTPCs quantification as a practical and clinically meaningful biomarker of aggressive disease biology in NDMM.

In conclusion, baseline CTPCs quantified in peripheral blood by next-generation flow cytometry stratified PFS in a real-world NDMM cohort, with the clearest separation at a pragmatic 0.05% cutoff. The cohort included both transplant-eligible and transplant-ineligible patients and substantial first-line daratumumab use, reflecting contemporary practice. CTPCs correlated with burden-related measures, supporting biological plausibility. Although the 0.02% cutoff proposed by the European CTC Consortium was not validated in this small cohort, our optimal 0.05% cutoff should only be viewed as exploratory and should not be interpreted as defining a new clinical threshold.

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Supplementary data are available in the online version of the paper.

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Figure Legends

Figure 1. Illustrative bivariate dotplots showing: A) 7.25% tumor plasma cells (PCs) (violet) and 0.24% normal PCs (green) in the bone marrow, and B) 0.019% tumor PCs (violet) and 0.067%

normal PCs (green) in the peripheral blood of the same patient with newly diagnosed multiple myeloma. The remaining cell populations are shown in grey.

Figure 2. Progression-free survival from diagnosis in NDMM patients after therapy: A) comparison of patients with ≤ 0.05% vs. > 0.05% tumor plasma cells (PCs) in peripheral blood (PB), B) comparison of patients with ≤ 0.02% vs. > 0.02% normal PCs in PB, C) comparison of patients with ≤ 1.69% vs. > 1.69% tumor PCs in bone marrow (BM), and D) comparison of patients with ≤ 0.06% vs. > 0.06% normal PCs in BM. Abbreviations: mPFS-median PFS expressed in months; p-statistical significance calculated using the log-rank test

Table 1. Clinico-biological characteristics of patients with newly diagnosed multiple myeloma (n=55).

Characteristic		Value
Age (years)	median (range)	70 (43-87)
Gender (n, %)	male	35 (63.6%)
	female	20 (36.4%)
Disease status (n, %)	newly diagnosed	55 (100%)
Bone marrow plasma cells by cytology (%)	median (range)	37.5 (4-95)
Monoclone size (g/l)	median (range)	20.3 (0.12-94.0)
R2-ISS stage (n, %)	I	6 (10.9%)
	II	14 (25.5%)
	III	30 (54.5%)
	IV	4 (7.3%)
High-risk cytogenetics (n, %)	no	21 (38.2%)
	yes	34 (61.8%)
Daratumumab-based 1st line therapy	no	30 (54.5%)
	yes	25 (45.5%)
ASCT performed	no	27 (49.1%)
	yes	28 (50.9%)
Response to therapy (n, %)	complete response (CR)	17 (30.9%)
	worse than CR	32 (58.2%)
PFS event (progression/death) (n, %)	no	34 (61.8%)
	yes	21 (38.2%)

Notes: The data on R2-ISS stage and the response to therapy were available in 54 and 49 patients, respectively. The remaining data were available in all patients.

Abbreviations: ASCT-Autologous Stem Cell Transplantation; PFS-Progression-Free Survival; R2-ISS-Second Revision of the International Staging System

519 **Table 2.** Levels of normal and tumor plasma cells in PB and BM of NDMM patients at diagnosis.

Proportion of	Mean	Median	Standard deviation	Range
Normal PCs in PB	0.0577%	0.0349%	0.0808%	0.0018-0.4381%
Tumor PCs in PB (CTPCs)	1.6674%	0.0159%	6.3983%	0.0006-44.2223%
Normal PCs in BM	0.0720%	0.0370%	0.0919%	0.0039-0.4477%
Tumor PCs in BM	18.8638%	8.2450%	23.0167%	0.0046-80.4207%

520 Abbreviations: BM-bone marrow; CTPCs-circulating tumor plasma cells; PB-peripheral blood;
521 PCs-plasma cells

Table 3. Correlations observed between variables related to tumor burden.

		% BM PCs by cytology	M-protein	%normal PCs in PB	% tumor PCs in PB (CTPCs)	% normal PCs in BM	% tumor PCs in BM
% BM PCs by cytology	ρ	1.000	.416**	.267	.595***	-.409**	.601***
	Sig. (2-tailed)		.004	.061	<.001	.004	<.001
M-protein	ρ		1.000	-.017	.377**	-.344*	.528***
	Sig. (2-tailed)			.907	.007	.015	<.001
%normal PCs in PB	ρ			1.000	.258	.443***	.222
	Sig. (2-tailed)				.057	<.001	.107
% tumor PCs in PB (CTPCs)	ρ				1.000	-.309*	.764***
	Sig. (2-tailed)					.023	<.001
% normal PCs in BM	ρ					1.000	-.427**
	Sig. (2-tailed)						.001
% tumor PCs in BM	ρ						1.000
	Sig. (2-tailed)						

Notes: *,**, *** correlation is significant at the 0.05, 0.01, 0.001 level, respectively

Abbreviations: BM-bone marrow; M-protein-monoclonal protein; PB-peripheral blood; PCs-plasma cells, ρ-Spearman's rank correlation coefficient

Table 4. Progression-free survival according to baseline CTPCs cutoffs.

CTPCs cutoff	High/low n	Events high/low	Median PFS high vs. low (months)	Log-rank p-value	HR (95% CI), p-value
> 0.02%	25/30	13/8	27.3 vs. 40.3	0.068	2.23 (0.92-5.42), p=0.075
> 0.05%	18/37	12/9	14.3 vs. 40.3	0.007	3.07 (1.29-7.31), p=0.011
> 0.07%	16/39	10/11	21.9 vs. 40.3	0.055	2.26 (0.96-5.33), p=0.062
> 2%	7/48	3/18	30.2 vs. 33.5	0.913	0.93 (0.27-3.21), p=0.913
> 5%	4/51	2/19	5.8 vs. 33.5	0.301	2.14 (0.49-9.31), p=0.312

Notes: CTPCs analyses were based on 55 evaluable patients with available baseline CTPCs percentage

Abbreviations: CTPCs-circulating tumor plasma cells; HR-hazard ratio; PFS-progression-free survival

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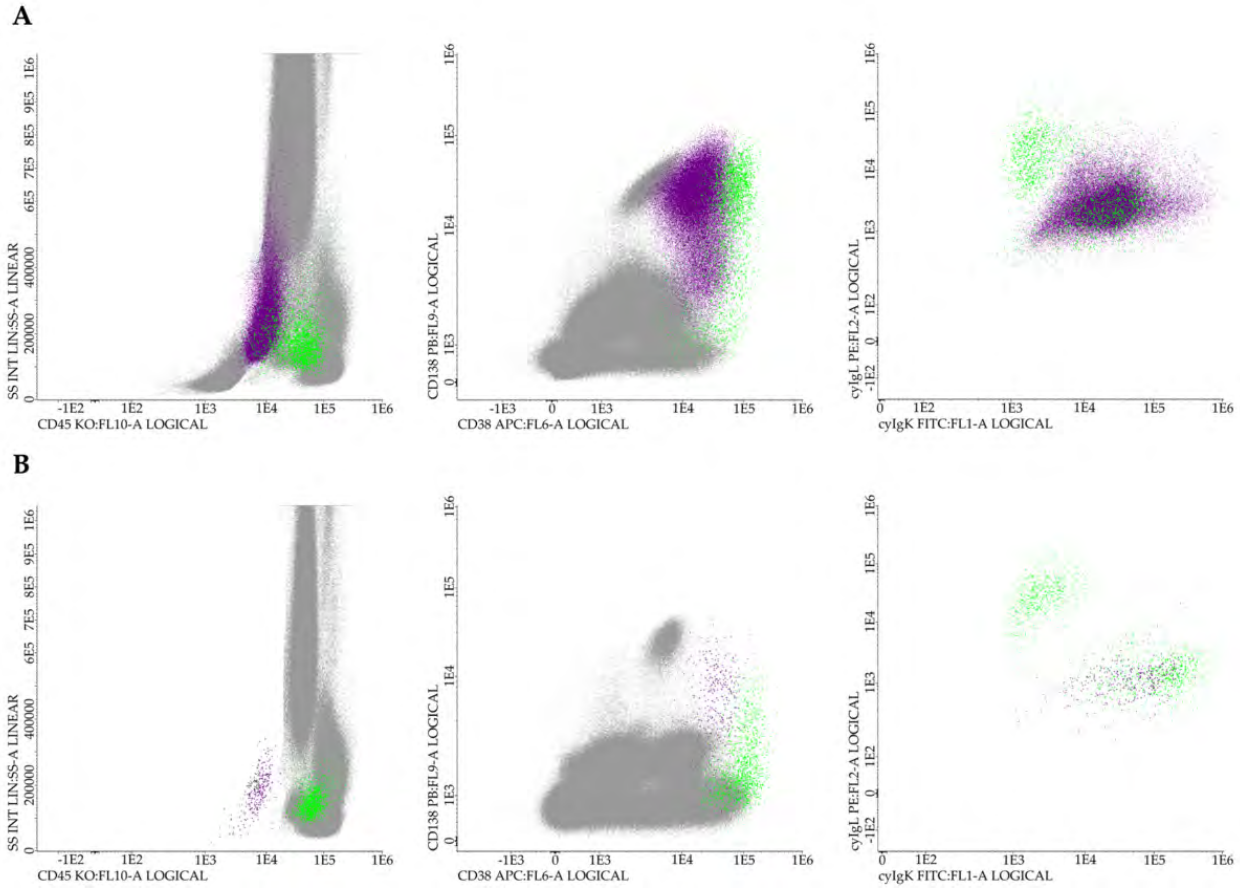

Fig. 2 Download full resolution image