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Prognostic value of antigen expression in multiple myeloma: a PETHEMA/GEM study on 1,265 patients enrolled in four consecutive clinical trials

Running title: Prognostic value of antigen expression in myeloma

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ABSTRACT

Persistence of minimal residual disease (MRD) after treatment for myeloma predicts inferior outcomes, but within MRD-positive patients there is great heterogeneity with both early and very late relapses. Amongst different MRD techniques, flow cytometry provides additional information about antigen expression on tumor cells, which could potentially contribute to stratify MRD-positive patients. We investigated the prognostic value of those antigens required to monitor MRD in 1,265 newly-diagnosed patients enrolled in the GEM2000, GEM2005MENOS65, GEM2005MAS65 and GEM2010MAS65 protocols. Overall, $CD19^{pos}$, $CD27^{neg}$, $CD38^{lo}$, $CD45^{pos}$, $CD81^{pos}$, $CD117^{neg}$ and $CD138^{lo}$ expression predicted inferior outcomes. Through principal-component-analysis, we found that simultaneous $CD38^{low}CD81^{pos}CD117^{neg}$ expression emerged as the most powerful combination with independent prognostic value for progression-free survival (HR:1.69; *P* =.002). This unique phenotypic profile retained prognostic value among MRD-positive patients. We then used next-generation flow to determine antigen stability throughout the course of the disease, and found that the expression of antigens required to monitor MRD is mostly stable from diagnosis to MRD stages, except for CD81 whose expression progressively increased from baseline to chemoresistant tumor cells (14% vs 28%). Altogether, we showed that the phenotypic profile of tumor cells provides additional prognostic information, and could be used to further predict risk of relapse among MRD-positive patients.

INTRODUCTION

In the past 15 years, treatment of multiple myeloma (MM) has improved dramatically due to progressively greater availability of active new drugs.(1–7) Accordingly, better techniques and biomarkers are needed to monitor treatment efficacy and predict outcome, in order to individualize therapy and avoid both under- and over-treating patients. Minimal residual disease (MRD) is emerging as an attractive biomarker to evaluate treatment efficacy and eventually, act as surrogate for superior outcomes based on significantly different MRD-negative rates.(8–10) Accordingly, the International Myeloma Working Group (IMWG) has recently reported new guidelines on response assessment that include criteria for MRD-negativity defined by next-generation sequencing (NGS) and flow cytometry (NGF), and has recommended their inclusion in clinical trials.(11) Both molecular (12) and immunophenotypic (13) techniques used to monitor MRD have recently benefited from methodological optimization leading to improved sensitivity (up to 10^{-6}), and better definition of MRD-negative patients with significantly lower probability of relapse. Conversely, the ability to detect ultra-low levels of MRD is increasing the percentage of MRD-positive patients which, due to a plethora of other clinical and biologic features, will include cases with shorter vs remarkably longer progression-free (PFS) and overall survival (OS).(10,14,15) Thus, improved risk stratification of MRD-positive patients emerges as un unmet area of research that could provide additional information to help tailoring subsequent treatment and monitoring.

One of the advantages of NGF is to afford real-time information on other parameters beyond MRD, due to both the need to perform the assay on fresh samples and the ubiquitous expression on other cells of those antigens required to detect MRD. This unique feature of NGF has recently shown to be useful to assess quality of bone marrow (BM) aspirates and exclude hemodiluted samples (13), define patient-specific immune signatures associated with different outcomes (15), and provide insight into the biology of ultra-chemoresistant MRD cells.(16,17) More recently, we have identified new patterns of antigen expression based on CD19 and CD81 that defined normal and tumor plasma cells (PCs) with distinct levels of differentiation, and helped revealing through longitudinal comparison of diagnostic vs. MRD samples, that in a subset of patients, less-differentiated PCs subclones become enriched after therapy-induced pressure.(18) Thus, it could be hypothesized that the phenotypic profile of clonal PCs assessed either at diagnosis or during MRD stages could also afford valuable information, particularly if we could demonstrate that those antigens required to monitor MRD remain stable throughout the course of disease, and therefore are hallmarks of residual PC clones. Unfortunately, there is limited data on the prognostic value of

antigen expression after the incorporation of proteasome inhibitors (PIs) and immune modulators (IMIDs) in MM (18–20), and almost no information on the stability of those antigens from diagnosis to MRD stages.(16,18)

Here, we started by investigating in a very large series of 1,265 newly-diagnosed MM patients enrolled into four consecutive PETHEMA/GEM protocols conducted before and after the incorporation of PIs and IMIDs, as well as in the transplant-eligible and -ineligible settings, the prognostic value of antigen expression at diagnosis. Afterward, we used NGF to compare in a cohort of 25 uniformly treated MRD-positive MM patients, the phenotypic profile of clonal PCs at diagnosis and MRD assessments after induction, intensification and consolidation. Overall, we defined a unique phenotypic profile with independent prognostic value, and that is rather stable from diagnosis to MRD stages. Accordingly, antigen expression of clonal PCs provides additional prognostic information that might potentially be used to further predict short vs long-term risk of relapse among MRD-positive patients.

PATIENTS AND METHODS

Patients and study designs. A total of 1,265 newly-diagnosed MM patients enrolled in four consecutive PETHEMA/GEM clinical trials (GEM2000 and GEM2005MENOS65 for transplant-eligible; GEM2005MAS65 and GEM2010MAS65 for elderly patients), and with available immunophenotypic data at diagnosis to investigate the prognostic value of antigen expression were included in this study. The design of the GEM2000 (NCT00560053) (21), GEM2005MENOS65 (NCT00461747) (22), GEM2005MAS65 (NCT00443235) (23) and GEM2010MAS65 (NCT01237249) (24) protocols has been described elsewhere. Because in most cases diagnostic and MRD studies were both performed with 4-color immunophenotyping, we took advantage of the prospective incorporation of 8-color NGF in the GEM2012MENOS65 clinical trial to investigate whether the phenotypic profile of clonal PCs at MRD assessment stages reflects that found at diagnosis (n=25), or shows significant therapy-induced modulation of antigens required to monitor MRD. According to the design of the GEM2012MENOS65 protocol (NCT01916252), MRD was assessed in all patients after six induction cycles with bortezomib, lenalidomide and dexamethasone (VRD) followed by high-dose therapy (HDT) with melphalan or melphalan plus busulfan and autologous stem cell transplantation (ASCT), and two courses of consolidation with VRD (NCT01916252). We randomly selected 25 patients with persistent MRD at all time-points to compare

the phenotypic profile of clonal PCs at diagnosis vs MRD after various stages of treatment. Baseline demographics and patient characteristics per clinical trial are provided in Table 1. All samples were collected after informed consent was given by each patient, according to the local ethical committees and the Helsinki Declaration. Median follow-up of the whole series was of 69 months.

Multiparameter flow cytometry. Erythrocyte-lysed whole BM samples from patients treated according to the GEM2000 protocol (n=479) were stained using a four-color direct immunofluorescence technique and the following monoclonal antibody (MoAb) [fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5), allophycocyanine (APC)] combinations were used to identify aberrant antigen expression in PCs: i) CD38, CD56, CD19, CD45; ii) CD138, CD28, CD33, CD38; and iii) CD20, CD117, CD138, CD38.(25) Conversely, patients included in the GEM2005MENOS65 (n=316) and GEM2005MAS65 (n=238) studies were stained with following MoAb combinations (FITC, PE, PerCP-Cy5.5, APC): i) CD38, CD56, CD19, CD45; ii) CD38, CD27, CD45, CD28; and iii) β2microglobulin, CD81, CD38, CD117.(20) Acquisition was performed in a FACSCalibur™ flow cytometer (BD Biosciences [BD], San Jose, CA) using the CellQUEST program (BD), and information was recorded for $3x10^3$ BMPC per combination. Regarding those patients enrolled in the GEM2010MAS65 clinical trial (n=232), approximately 200µL of EDTA-anticoagulated BM aspirated samples were immunophenotyped using two different 8-color combinations of MoAb - [Pacific Blue, Pacific Orange, FITC, PE, PerCP-Cy5.5, PE-cyanine 7 (PE-Cy7), APC, APCH7]: i) CD45, CD138, CD38, CD56, β2microglobulin, CD19, cyKappa, cyLambda; ii) CD45, CD138, CD38, CD28, CD27, CD19, CD117, CD81 – following the EuroFlow guidelines and the source of antibodies to identify clonal PCs, and characterize their phenotypic profile.(26) Data acquisition was performed for approximately 10^6 leukocytes/tube in a FACSCantoII flow cytometer (BD, San Jose, CA) using the FACSDiva 6.1 software (BD). Data analysis was performed using the Infinicyt software (Cytognos SL, Salamanca, Spain).

Next-generation flow cytometry. We used NGF for real-time and sensitive MRD monitoring of patients enrolled in the GEM2012MENOS65 study (n=25). Briefly, the method is based on a (standardized) lyse-wash-and-stain sample preparation protocol that allows the measurement of high numbers of bone marrow (BM) cells ($2x \geq 5x10^6$ cells/tube), and an optimized 8-color, 2-tubes, antibody panel, for accurate identification of BM PCs and discrimination between phenotypically aberrant and normal PCs: tube 1: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-

PerCPCy5.5, CD19-PECy7, CD117-APC, CD81-APCH7 and; tube 2: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, cyKAPPA-APC, cyLAMBDA-APCH7. The two-tube strategy allows detection of MRD with specific confirmation of monoclonality (through light-chain restriction) on phenotypically aberrant PCs, identified either by antigen under-expression (CD19, CD27, CD38, CD45, CD81) or antigen over-expression (CD56, CD117, CD138). Based on optimal source of antibodies, novel software tools for automatic PC gating and fusion of data corresponding to the two different aliquots/tubes described above using the Infinicyt ™ software (Cytognos, Spain) (13), the median limit of MRD detection was of $2x10^{-6}$.

Fluorescence in Situ Hybridization (FISH) studies. Interphase FISH was performed on immunomagnetic-enriched PC at diagnosis (n=777). Patients were tested for t(4;14), t(14;16) and del(17p), amongst others. Those cases displaying any of the above were classified as having high-risk FISH, whereas all the others as standard-risk patients.

Statistical analysis. PFS was measured from time of diagnosis to progression or death from any cause. OS was defined as time from diagnosis to death from any cause. Survival was analyzed by the Kaplan–Meier method, and differences between curves were tested for statistical significance with the (two-sided) log-rank test. A univariate analysis was conducted to assess the impact on PFS and OS of transplant-eligibility, as well as various baseline prognostic factors including, the International Staging System (ISS), serum LDH levels, and FISH cytogenetics. A multivariate Cox proportional hazard model was developed to explore the independent effect on PFS and OS of variables with significant impact on the univariate analysis. Multivariate analysis and visualization of phenotypic and cytogenetic profiles was performed based on antigen expression profiles scaled from 0 to 100, using the t-SNE algorithm implemented in the Infinicyt software. For longitudinal analysis and comparison of quantitative and qualitative parameters related to the phenotypic profile of clonal PCs at diagnosis and at different MRD time-points, the Friedman and ANOVA tests for repeated measures with the Greenhause-Geisser correction factor were used, respectively. A P value <.05 was considered statistically significant. The SPSS software (version 20.0; IBM, Chicago, IL, USA) and the R environment software (version 3.2.3, The R Foundation for Statistical Computing) were used for statistical analyses.

RESULTS

Prognostic value of antigen expression in multiple myeloma. We first investigated the prognostic value of those antigens required to monitor MRD in a large series of 1,265 newly-diagnosed patients enrolled in the GEM2000 and GEM2005MENOS65 protocols for transplant-eligible, and the GEM2005MAS65 and GEM2010MAS65 protocols for elderly MM. Patients were dichotomized based on the percentage of clonal PCs expressing markers with bimodal patterns of expression (i.e.: CD19, CD27, CD45, CD56, CD81 and CD117), or based on the mean fluorescence intensity (MFI) in clonal PCs vs other nucleated cells of markers with unimodal patterns of expression (i.e.: low vs bright staining of CD38 and CD138). For the former set of markers, optimal expression cut-offs were defined based on the closest percent quartile to which the lowest *P* -value was reached for PFS (i.e.: 0%, 25%, 50%, 75% or 100% of clonal PCs expressing the marker). Pooled analysis of the 1,265 patients under study showed prognostic value for all markers analyzed except CD56 (Table 2). However, detailed analyses according to the type of treatment revealed that the negative impact of CD19 (when positive in ≥50% clonal PCs) and, particularly, CD45 expression (when positive in ≥25% clonal PCs) was only significant in elderly patients (median PFS of 22 vs 32 months, *P* =.001; median OS of 43 vs 60 months; *P* =.016). Conversely, the favorable effect of CD117 expression (when positive in ≥50% clonal PCs) was only significant in transplant-eligible patients treated without PIs and/or IMIDs (median PFS of 44 vs 32 months, *P* =.004; median OS of 88 vs 52 months; *P* <.001). In turn, the patterns of expression of CD38, CD81 and CD138 were of prognostic value in all therapeutic protocols these markers were analyzed (Table 2). Patients with aberrantly low CD38 MFI had inferior PFS (median of 30 vs 38 months; *P* <.001) and OS (median of 58 vs 77 months; *P* <.001) vs cases with bright CD38 staining; comparable results were observed in patients with aberrantly low CD138 expression (median PFS of 29 vs 34 months, *P* =.003; median OS of 55 vs 67 months, *P* =.04) compared to cases with bright CD138 staining. In turn, presence of ≥25% CD81+^{ve} clonal PCs identified a subset of patients with inferior PFS (median of 24 vs 38 months; *P* <.001) and OS (median of 53 vs 81 months; *P* <.001). These results showed that most markers required to monitor MRD are of prognostic relevance in MM, but also that treatment intensification together with the use of more effective drugs, may modulate the prognostic impact of selected antigens.

Defining a phenotypic profile to predict outcomes at diagnosis and MRD stages. Since many of the markers that are used to monitor MRD showed prognostic value, we

then used principal component analysis (PCA) to test all possible antigen combinations and unbiasedly define the overall phenotypic profile related with the greatest difference in patients' survival. Thus, aberrantly low expression of CD38 together with reactivity for CD81 (≥25% positive PCs) in the absence of homogeneous expression of CD117 (<50% positive PCs) identified a subset of patients (n=100; 9%) that compared to the remaining cases, had a significantly inferior PFS (median of 22 vs 35 months; *P* <.001) and OS (median of 43 vs 76 months; *P* <.001) (Figure 1A-B). The $CD38^{low}CD81^{+}CD117^{-}$ phenotypic profile was also related to significantly higher rates of early relapse (i.e.: ≤12 months after diagnosis) as compared to the rest of cases (28% vs 17%; *P* =.011). Multivariate analysis of baseline prognostic factors for survival, including the phenotypic profile of clonal PCs plus patients' eligibility for HDT/ASCT, ISS, LDH and FISH cytogenetics, together with patients' MRD status after treatment, showed that the $CD38^{low}CD81^{+}CD117^{-}$ phenotypic profile had an independent adverse effect in patients' PFS (hazard ratio -HR-: 1.69; *P* =.002) as well as a trend for OS (HR: 1.46; *P* =.093) (Table 3). Of note, despite significant associations were found between the expression of individual antigens and the presence of individual cytogenetic abnormalities (Supplementary Table 1), tSNE projection of the global phenotypic profiles of clonal PCs revealed that patients with high-risk FISH abnormalities – e.g.: t(4;14), t(14;16), and/or del(17p) - do not clustered together (Figure 2). Thus, patients with the adverse phenotypic profile defined above did not show a significantly higher frequency of high-risk FISH abnormalities vs all other cases (20% vs 19%; *P* =.5). Lastly, we investigated whether the $CD38^{low}CD81^{+}CD117^{-}$ adverse phenotypic profile was also observed among patients with persistent MRD after treatment (n=396). As compared to all other MRD-positive patients, those cases displaying the $CD38^{low}CD81^{+}CD117^{-}$ adverse phenotypic profile (n=45; 11%) had significantly shorter PFS (median of 24 vs 36 months; *P* =.002) and a trend towards inferior OS (median of 53 vs 74 months; *P* =.058) (Figure 1C-D). Of note, MRD levels were not significantly different between MRD-positive patients displaying the $CD38^{low}CD81^{+}CD117^{-}$ adverse phenotypic profile vs all other cases (median of 0.22% vs 0.11%; *P* =.67).

Longitudinal comparison of phenotypic profiles of tumor plasma cells from diagnosis to MRD stages. Currently, data about antigen stability from diagnosis to MRD stages in MM is very limited, which prompted us to compare the antigen expression profile of clonal PCs at diagnosis and after various stages of therapy in a cohort of 25 MRD-positive patients homogenously treated according to the GEM2012MENOS65 protocol, and investigate whether the phenotypic profile of MRD clonal PCs after therapy represents that found at the time of diagnosis. In such case,

the presence of the unique phenotypic profile here identified, at diagnosis, would predict for more resistant MRD clones and adverse prognosis. As expected, tumor burden significantly decreased from diagnosis to MRD assessments after six cycles of induction with VRD, HDT/ASCT and two courses of VRD consolidation (median percentage of clonal PCs of 6%, 0.05%, 0.009% and 0.003%, respectively; P <.001) (Figure 3A). Thus, therapy-induced pressure over total clonal PCs at diagnosis led to the selection and persistence of very small numbers of chemoresistant MRD clonal PCs that, compared to patient-paired diagnostic PC clones, showed progressively higher expression of CD81 (mean percentage of positive clonal PCs at diagnosis, after induction, HDT/ASCT and consolidation of 14%, 15%, 22% and 28%; P =.002). In contrast, no significant differences were noted on the levels of expression of CD38 and CD138, nor the percentage of clonal PCs expressing CD19, CD27, CD45, CD56 and CD117 (Figure 3B). Overall, shifts in antigen expression (i.e.: from negative to ≥20% of positive clonal PCs or vice-versa) for any of the six markers under study which showed bimodal expression were observed in only 13 out of the 75 (17%) MRD BM samples analyzed (e.g. in a patient with double-antigen shift illustrated in Figure 3C). Thus, these results indicate that the overall phenotypic profile of clonal PCs from MM patients at diagnosis remains largely stable at different MRD stages, and that the potential prognostic value of antigen expression assessed after treatment in chemoresistant cells could be inferred from that found at diagnosis (Supplementary Figure 1).

DISCUSSION

The relevance of MRD monitoring in MM has significantly increased in recent years, evolving from a biomarker used to assess tumor contamination of stem cell harvests into one of the best predictors of outcome and, eventually, a surrogate of PFS and OS.(27) In parallel, traditional immunophenotypic and molecular methods have been replaced by more sensitive next-generation techniques, recently adopted by the IMWG to define NGF- and NGS-based MRD negative criteria.(11) Here, we investigated the prognostic value of those immunophenotypic markers required to monitor MRD by flow cytometry in one of the largest series of newly-diagnosed MM patients enrolled into four consecutive Spanish protocols; simultaneous expression of a CD38^{low}CD81^{+}CD117^{-} phenotypic profile was identified as the most powerful combination to discriminate patients with inferior outcomes, and it provided additional information to stratify risk among patients with persistent MRD.

In this study, we also provide novel and practical information for those using NGF to monitor MRD in MM. In acute myeloid leukemia, it is well-known that the immunophenotype of the leukemic clone may change between diagnosis and relapse due to subclonal selection/evolution, which has important consequences for MRD detection, due to potential false-negative results after disappearance of aberrant phenotypes used as MRD markers.(28) Here, we showed for the first time that, for different treatment stages, shifts in antigen expression during MRD assessment take place in a small subset of MM patient samples (17%), and mostly involving CD81. Thus, while information about the patients' phenotype at diagnosis might be helpful to monitor MRD, it should not be misleading given the possibility of antigen instability from diagnosis to MRD stages in a few patients. However, changes in one particular antigen do not affect MRD detection in MM, which is based in the assessment of multiple phenotypic aberrancies that are detected by using multidimensional (≥8) and optimal combination of mAbs; in fact, it has been recently shown that it is possible to detect clonal PCs irrespectively of the patient-specific phenotypic aberrancies.(13) This allows the use of NGF-based MRD assessment, even in the absence of diagnostic samples, in virtually all MM patients.(13) Furthermore, the two-tube NGF approach also allows confirmation in a second independent measurement of the clonal nature of phenotypically aberrant PCs, through evaluation of cytoplasmic κ/λ immunoglobulin light-chain restriction.(13) Thereby, as shown here, the capability of detecting MRD remains identical in patients with or without antigen stability from diagnosis to different MRD stages.

Despite the use of highly effective drug combinations, next-generation MRD techniques have recently demonstrated that a great fraction of the patients still remains MRD positive after treatment.(12,13,29) Interestingly however, whereas with conventional therapies the prognosis of MRD-positive patients has been typically adverse (10,30,31), more recent studies, particularly those conducted after the incorporation of daratumumab revealed that the outcome of some MRD-positive patients can be remarkably improved.(32) Whenever such monoclonal antibody-based therapies will become a mainstay in the treatment of MM, it would thereby become important to identify other markers capable of discriminating among MRD-positive patients, those that may reach long-term survival vs those at risk of short-term progression. We and others had previously reported that the presence of high-risk cytogenetics at baseline identifies a subset of MRD positive patients with dismal survival (10,14,15,33); here, we describe that simultaneous to MRD assessment, NGF could also provide additional prognostic information based on the phenotypic profile of

clonal PCs; thus, patients displaying a CD38^{low}CD81^{+}CD117^{-} antigen expression profile would be at risk of significantly shorter survival. It should be noted that through t-SNE projection no clear correlation between the presence of high-risk cytogenetic abnormalities and such unique phenotypic profile was found; thus, information on the later would be complementary to improve risk stratification of MRD-positive patients.

One limitation of the present study was the usage of different monoclonal antibody conjugates from older into more recent clinical trials. However, it should be noted that either the percentage or MFI of marker expression on clonal PCs was compared vs other nucleated cells in each individual sample, and not longitudinal across trials. Because interpretation of marker expression is thus based on the sample internal controls (i.e.: other BM nucleated cells), it is possible to pool data from different clinical trials for comprehensive analyses about the prognostic value of antigen expression. The favorable prognosis of MM patients with CD117^{+} expression has been hypothesized due to an altered homing of clonal PCs in the BM towards neutrophil precursor niches, thereby contributing to a greater maintenance of residual normal PCs.(34) Taking advantage of the large series of patients included in this study treated according to four different treatment protocols, we observed that when analyzed individually, the pattern of CD117 expression loses prognostic significance after PIs and IMIDs have been introduced. These findings could be related to the ability of these drugs to disturb the tumor microenvironment. Most recently, we demonstrated that MM patients harboring less-differentiated PCs, defined in part by CD81^{+} expression, had dismal survival, could be related to the higher chemoresistant potential of less differentiated PC clones.(18) Here, we confirm previous observations about the prognostic role of CD81 in MM (18,20), and showed that similarly to what has been described in transplant-ineligible patients, less-differentiated PC subclones also become significantly enriched after HDT/ASCT. Furthermore, a CD38^{low} CD45+ CD81+ phenotypic profile emerged as the most powerful to identify a small subset of patients (n=29, 2,3%) out of which almost half relapsed in the first 12 months after diagnosis (46% vs 18% in the rest of patients; P <.001). Based on recent data suggesting a potential role for the *all-trans* retinoic acid in improving the efficacy of daratumumab *via* up-regulation of CD38 expression (35), it might be worth to investigate whether drugs able to induce cellular differentiation could improve the outcome of patients with less-differentiated CD81^{+} clonal PCs. Conversely, the phenotypic profile of MRD cells could also potentially contribute to optimize the use of specific immunotherapies targeting surface antigens. CD38 is a multifunctional cell surface glycoprotein that serves as both a receptor for the transduction of activation/proliferation signals and an

ectoenzyme that catalyzes the production of nucleotides involved in calcium signaling.(36) However, given the end-stage long-lived and quiescent phenotype of CD38^{bright} PCs vs CD38^{low} plasmablasts, it could be hypothesized that rather than signaling, CD38 acts as a marker of migration and adhesion properties involved in anchoring PCs to the BM stroma.(37,38) Interestingly, we and others have reported that like normal peripheral blood PCs, MM circulating tumor cells also show lower levels of surface CD38 and CD138 vs their BM clonal PCs counterpart.(39,40) Here, we confirm on preliminary data (41) indicating that MM patients with CD38^{low} and CD138^{low} expression have significantly inferior PFS and OS as compared to patients with normal (bright) CD38 levels. In fact, the CD138^{high}CD38^{high}CD19- phenotypic profile emerged as the most powerful combination to define a subset of patients (n=299; 34%) that compared to the rest of cases, displayed significantly superior PFS (median of 39 vs. 30 months; P <.001) and OS (median of 71 vs 59 months; P <.001). Furthermore, this phenotypic profile remained significant even MRD-positive patients; those cases displaying the CD138^{high}CD38^{high}CD19- phenotypic profile (n=90; 32%) had significantly longer PFS (median of 37 vs 32 months; P =.017) and OS (median of 81 vs 65 months; P =.007). Since recent data obtained in a phase I study suggested that patients with lower CD38 expression showed a smaller benefit after treatment with daratumumab (42), further investigation in larger series of patients are warranted to understand if the use of different immunotherapy modalities can be tailored according to the density of antigen target molecules on the surface of clonal PCs.

Due to the progressively higher efficacy of novel drug combinations, there is risk for persistent MRD to be generally considered as a treatment failure, even though not all MRD-positive patients are at high-risk of progression and some may even reach long-term survival.(10) Overall, this study shows that phenotypic characterization of clonal PCs could be of added value to stratify risk among patients with persistent MRD by sensitive next-generation MRD techniques. Further studies based on modern treatment regimens and with a redefined MRD-positive patient population based on the higher sensitivity of NGF, are warranted to establish the role of phenotypic characterization of MRD clonal PCs.

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Figure 1. Defining a phenotypic profile to predict outcomes. We used R environment to test all possible antigen combinations and define the phenotypic profile related with the greatest difference in patients' survival. Accordingly, aberrantly low expression of CD38 together with reactivity for CD81 (≥25% positive PCs) in the absence of homogeneous expression of CD117 (<50% positive PCs) identified a subset of patients that compared to the rest of cases, had significantly inferior progression-free and overall survival both when analyzed in all patients (A-B) as well as in those with persistent MRD after treatment (n=396; C-D).

Figure 2. Correlation between phenotypic and cytogenetic profiles. t-Distributed Stochastic Neighbor Embedding (t-SNE) projection of the patients' phenotypic profiles defined by antigen expression in PCs at the time of diagnosis (n=672). Patients without any high-risk cytogenetic abnormality [i.e.: t(4;14), t(14;16) and/or del(17p)] are represented by grey circles (n=544), whereas patients with t(14;16) and del(17p) (n=2), t(4;14) and del(17p) (n=9), del(17p) (n=44), t(4;14) and t(14;16) (n=2), t(14;16) (n=9), and t(4;14) (n=61) are highlighted as in blue, pink, dark-green, red, light-green, and brown squares, respectively.

Figure 3. Longitudinal analysis of phenotypic profiles on clonal plasma cell (PCs) analyzed at diagnosis vs MRD stages. (A) Percentage of bone marrow (BM) clonal PCs at diagnosis and during minimal residual disease (MRD) monitoring after 6 induction cycles with bortezomib, lenalidomide, dexamethasone (VRD), high-dose therapy followed by autologous stem cell transplantation (HDT/ACST) and 2 courses of VRD consolidation, according to the GEM2012MENOS65 clinical trial (n=25). Dots and vertical lines represent median values and 25th-75th percentiles, respectively. **(B)** Comparison of clonal PCs phenotypic profiles was performed in longitudinal samples available at all timepoints. Antigen expression is represented as the percentage of clonal PCs expressing the marker, or as mean fluorescence intensity (MFI) for markers with unimodal expression (i.e.: CD38 and CD138). Each dot represents an individual patient. **(C)** Bivariate dot-plots of a patient representative of shifts in antigen expression on clonal PCs from diagnosis to MRD stages (e.g.: CD81 and CD117). The percentage of BM clonal PCs in each timepoint is indicated between brackets.