

## Biological and clinical significance of dysplastic hematopoiesis in patients with newly-diagnosed multiple myeloma

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### Abstract:

Risk of developing myelodysplastic syndromes (MDS) is significantly increased in both multiple myeloma (MM) and MGUS, suggesting that is therapy independent. However, the incidence and sequelae of dysplastic hematopoiesis at diagnosis are unknown. Here, we used multidimensional flow cytometry (MFC) to prospectively screen for presence of MDS-associated phenotypic alterations (MDS-PA) in the bone marrow of 285 MM patients enrolled in the PETHEMA/GEM2012MENOS65 trial (NCT01916252), and investigated the clinical significance of monocytic MDS-PA in a larger series of 1,252 patients enrolled in four PETHEMA/GEM protocols. At diagnosis, 33/285 (11.6%) cases displayed MDS-PA. Bulk- and single-cell targeted sequencing of MDS recurrently mutated genes in CD34+ progenitors (and dysplastic lineages) from 67 patients unveiled clonal hematopoiesis in 13/26 (50%) cases with MDS-PA versus 9/41 (22%) without MDS-PA; *TET2* and *NR4A5* were the most frequently mutated genes. Dynamics of MDS-PA at diagnosis and after autologous transplant were evaluated in 86/285 patients, and showed that in most cases (69/86, 80%) MDS-PA either persisted or remained absent in patients with or without MDS-PA at diagnosis, respectively. Noteworthy, MDS-associated mutations unfrequently emerged after high-dose therapy. Based on MFC profiling, we found that patients with

MDS-PA have altered hematopoiesis and Treg distribution in the tumor microenvironment. Importantly, presence of monocytic MDS-PA at diagnosis anticipated greater risk of hematological toxicity and was independently associated with inferior progression-free (HR:1.5,  $P=.02$ ) and overall survival (HR:1.7,  $P=.01$ ). This study unveils the biological and clinical significance of dysplastic hematopoiesis in newly-diagnosed MM, which can be screened with moderate sensitivity using cost-effective MFC.

**Conflict of interest:** COI declared - see note

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**KEY POINTS**

- Approximately 1 out of 10 patients with myeloma displays MDS-associated phenotypic abnormalities at diagnosis and have inferior survival
- MDS-associated phenotypic abnormalities modify the tumor microenvironment and induce greater risk of hematological toxicity from treatment

**ABSTRACT**

Risk of developing myelodysplastic syndromes (MDS) is significantly increased in both multiple myeloma (MM) and MGUS, suggesting that is therapy independent. However, the incidence and sequelae of dysplastic hematopoiesis at diagnosis are unknown. Here, we used multidimensional flow cytometry (MFC) to prospectively screen for presence of MDS-associated phenotypic alterations (MDS-PA) in the bone marrow of 285 MM patients enrolled in the PETHEMA/GEM2012MENOS65 trial (NCT01916252), and investigated the clinical significance of monocytic MDS-PA in a larger series of 1,252 patients enrolled in four PETHEMA/GEM protocols. At diagnosis, 33/285 (11.6%) cases displayed MDS-PA. Bulk- and single-cell targeted sequencing of MDS recurrently mutated genes in CD34+ progenitors (and dysplastic lineages) from 67 patients unveiled clonal hematopoiesis in 13/26 (50%) cases with MDS-PA versus 9/41 (22%) without MDS-PA; *TET2* and *NRAS* were the most frequently mutated genes. Dynamics of MDS-PA at diagnosis and after autologous transplant were evaluated in 86/285 patients, and showed that in most cases (69/86, 80%) MDS-PA either persisted or remained absent in patients with or without MDS-PA at diagnosis, respectively. Noteworthy, MDS-associated mutations unfrequently emerged after high-dose therapy. Based on MFC profiling, we found that patients with MDS-PA have altered hematopoiesis and Treg distribution in the tumor microenvironment. Importantly, presence of monocytic MDS-PA at diagnosis anticipated greater risk of hematological toxicity and was independently associated with inferior progression-free (HR:1.5,  $P=0.02$ ) and overall survival (HR:1.7,  $P=0.01$ ). This study unveils the biological and clinical significance of dysplastic hematopoiesis in newly-diagnosed MM, which can be screened with moderate sensitivity using cost-effective MFC.

## INTRODUCTION

Patients with multiple myeloma (MM) are living longer with increasingly effective therapies, but long-term complications including second primary malignancies (SPMs) emerged as an important challenge.<sup>1-4</sup> Accordingly, a large meta-analysis confirmed that patients who received lenalidomide and oral melphalan had an increased risk of developing hematological SPMs.<sup>5</sup> In the majority of cases these were myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML).<sup>6</sup>

Increased risk of MDS and AML following MM has been acknowledged for many decades.<sup>7</sup> In a Swedish population-based study, patients with MM had an 11.5-fold risk of developing MDS/AML that remained stable between 1986 and 2005, suggesting that such risk was present before the introduction of high-dose melphalan and immunomodulatory drugs (IMiDs).<sup>7</sup> Furthermore, an excess of MDS/AML was observed in subjects with MGUS who had never developed MM and therefore, had not received anti-MM therapy. Another population-based analysis of 605 patients with MGUS and 16,710 controls living in the Olmsted County<sup>8</sup>, confirmed an increased risk of developing MDS (though not AML) following MGUS. Thus, patients with benign plasma cell (PC) disorders also have intrinsically high-risk of developing MDS. Interestingly, both studies unveiled that subjects with IgM MGUS (where clonality is mostly confined to the B cell compartment rather than PCs)<sup>9</sup> had no significantly increased risk of developing MDS. These findings suggest a potential link between the emergence of clonal PCs in the bone marrow (BM) and progressive dysplasia. Notwithstanding, most studies have focused on the clinical diagnosis of MDS after therapy without investigating whether dysplastic features are already detectable prior the onset of MDS and, eventually, before initiating treatment.

Barlogie et al demonstrated a decade ago, the presence of MDS-associated cytogenetic abnormalities (MDS-CA) after high-dose therapy (HDT) in 4% of MM patients.<sup>10</sup> Afterwards, the Arkansas group showed that these cytogenetic alterations often precede clinical MDS and/or AML.<sup>11</sup> More recent studies used next-generation sequencing (NGS) to demonstrate the presence of clonal hematopoiesis in the apheresis of 5% of MM patients, prior to autologous stem cell transplantation (ASCT).<sup>12</sup> Preliminary studies based on multidimensional flow cytometry (MFC) have shown the presence of MDS-associated phenotypic abnormalities (MDS-PA) in up to 13% of patients with MM, and that a significant proportion of these cases with either multiple or even isolated MDS-PA at diagnosis may display genetic and/or morphologic evidences of clonal hematopoiesis.<sup>13,14</sup> However, if dysplastic hematopoiesis at the time of diagnosis has clinical consequences remains unknown; if it does, it could affect a considerable number of MM patients given their median age at diagnosis (70 years)<sup>15</sup>

and the high incidence of clonal hematopoiesis of indeterminate potential (CHIP) in elderly individuals.<sup>16</sup>

Here, we unveil that MDS-PA are detectable in approximately 1 out of 10 patients with newly-diagnosed MM, which is often associated with underlying clonal hematopoiesis that usually persist after HDT/ASCT. Strikingly, patients carrying MDS-PA are at greater risk of developing hematological toxicity during treatment and show inferior survival unless IMiDs are used during maintenance.

## PATIENTS AND METHODS

**Study design.** Screening of MDS-PA was performed prospectively in 285 patients with newly diagnosed MM, enrolled in the PETHEMA/GEM2012MENOS65 clinical trial (NCT0191652). This open-label phase 3 study included six induction cycles of bortezomib, lenalidomide and dexamethasone (VRD), autologous stem cell transplant (ASCT) conditioned with Bu-Mel or Mel-200 high-dose therapy (HDT), and two consolidation cycles of VRD. Afterwards, patients were enrolled in the PETHEMA/GEM2014MAIN clinical trial (NCT02406144) that randomized maintenance therapy with RD or RD plus ixazomib for two years, after which patients continued maintenance with RD for three additional years if positive for minimal residual disease (MRD) or stopped maintenance if MRD negative. In addition, monocytic MDS-PA were retrospectively evaluated in 967 patients enrolled in three PETHEMA/GEM clinical trials that included maintenance therapy: GEM2000 (N = 475) and GEM2005MENOS65 (N = 275) for transplant-eligible patients, and GEM2005MAS65 (N = 217) for transplant-ineligible. Study designs have been described elsewhere<sup>17–19</sup> and are illustrated in Supplemental Figure 1, which also includes the median follow-up of each protocol. Each study site's Independent Ethics Committee approved the protocol and informed consent forms, (required) prior to patient enrollment. All studies were conducted per the ethical principles of the Declaration of Helsinki.

**Screening of MDS-associated phenotypic abnormalities (MDS-PA).** MDS-PA were prospectively evaluated in the GEM2012MENOS65 clinical trial, within 24 hours after collection of EDTA-anticoagulated bone marrow (BM) aspirates. Briefly, the EuroFlow lyse-wash-and-stain sample preparation protocol (adjusted to  $10^6$  nucleated cells) together with the first combination of the EuroFlow next-generation flow (NGF) antibody panel (CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, CD81-APCH7)<sup>20</sup> were used for assessment of PC clonality<sup>21</sup> and also to quantify the percentage of CD56 positive monocytes. A monocytic MDS-PA was defined if  $\geq 50\%$  of total monocytes expressed CD56 (Supplemental Figure 2). An additional combination – HLADR-PacB, CD45-OC515, CD36-FITC, CD13-PE, CD34-PerCPCy5.5, CD117-PECy7, CD11b-APC, CD71-APCH7 – was specifically designed to assess MDS-PA in the neutrophil and erythroid lineages, based on the detection of altered maturation phenotypic pathways as described elsewhere (Supplemental Figure 2).<sup>13,14</sup> In 84 of the 285 patients enrolled in the GEM2012MENOS65 trial and screened for MDS-PA at diagnosis, an additional evaluation (using both monoclonal antibody combinations described above) was performed in BM aspirates collected for response assessment at day 100 after

HDT/ASCT, whenever sufficient sample was available after performing NGF-MRD assessment as described elsewhere.<sup>22</sup> In 47 of the 285 patients, another evaluation was performed during maintenance. An additional series of 59 consecutive newly-diagnosed MM patients was analyzed to validate potential associations between presence of MDS-PA and altered hematopoiesis. Data acquisition was performed in a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using the FACSDiva 6.1 software (BD). Data analysis was performed using the Infinicyt software (Cytognos SL, Salamanca, Spain). Monocytic MDS-PA were also evaluated in patients enrolled in the GEM2000, GEM2005MENOS65 and GEM2005MAS65 clinical trials, based on the 4-color combination - CD38-FITC, CD56-PE, CD19-PerCPCy5.5, CD45-APC - used for assessment of clonality at diagnosis.<sup>23</sup>

**Profiling the tumor microenvironment.** Based on the antibody combination defined above (HLADR-PacB, CD45-OC515, CD36-FITC, CD13-PE, CD34-PerCPCy5.5, CD117-PECy7, CD11b-APC, CD71-APCH7) and a T cell oriented combination evaluated in 42 patients after HDT/ASCT (CD4-PacB, PD1-BV510, CD45RA-FITC, CD127-PE, CD8-PerCPCy5.5, TCR $\gamma\delta$ -PECy7, CD25-APC, CD197-APCH7), principal component analyses (PCA) were performed to unbiasedly define all cellular clusters that were identifiable with these antibody combinations, and compare their distribution in the BM tumor microenvironment of patients with or without MDS-PA. Namely, the first combination allowed the systematic identification of 14 different cell-subsets whereas the second antibody combination allowed the systematic identification of 14 different T cell-subsets. In addition, expression levels of CD127 and PD-1 were evaluated in each T cell subset. Data acquisition was performed in a FACSCanto II flow cytometer and data analysis was performed using the Infinicyt software.

**Fluorescence activating cell sorting (FACS).** To establish the relationship between the presence of MDS-PA and clonal hematopoiesis, we used a FACSria II flow cytometer (BD) to sort CD34+ hematopoietic progenitor cells (HPCs) from 67 patients (40 enrolled in the GEM2012MENOS65 trial) with (N = 26) or without MDS-PA (N = 41). In the former, we also isolated the corresponding cell lineage(s) with MDS-PA (Figure 1A).

**Next-generation sequencing (NGS).** The TruSight™ myeloid targeted sequencing panel (Illumina, USA) and a customized PAN-Myeloid panel (SOPHiA GENETICS (Saint Sulpice, Switzerland) were used to detect MDS-associated single nucleotide variants (SNV) and indels in 40 patients enrolled in the GEM2012MENOS65 trial and



23 additional cases included to increase the sample size, respectively (Supplemental Tables 1 and 2). DNA was extracted using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and quantified using Qubit dsDNA BR Assay Kit Qubit 2.0 Fluorometer and (Life Technologies, Carlsbad, CA, USA) and DNA quality was assessed by DNA genomic kit on Tape Station 4100 (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were prepared from 50 ng of DNA per sample, following manufacturer's instructions, as described previously<sup>24</sup>. All samples were sequenced in an Illumina MiSeq with a depth equal or greater than 250x in 80% of bases with the TruSight™ myeloid targeted sequencing panel and 1000x in 99.66% of bases with the PAN-Myeloid panel. Presence of clonal hematopoiesis was assessed by single-cell DNA sequencing (scDNAseq) in four patients with MDS-PA (Supplemental Methods and Supplemental Table 3).

**Variant calling.** NGS data was processed using the bioinformatics software HD Genome One (DREAMgenics, Oviedo, Spain), certified with IVD/CE-marking.<sup>25</sup> Briefly, raw FASTQ files were evaluated using quality control checks from FastQC<sup>26</sup> and Trimmomatic<sup>27</sup> was employed to remove low quality bases, adapters and other technical sequences. Each FASTQ file was aligned to the human reference genome (GRCh37/hg19) using BWA-mem<sup>28</sup>, generating sorted BAM files with SAMtools.<sup>29</sup> Reads from the same libraries were then merged. As samples were processed using an amplicon panel, optical and PCR duplicates were not removed. SNVs and indels were identified using a variation of the Sidrón algorithm<sup>30</sup> with the following parameters: total read depth  $\geq 6$ , mutated allele count  $\geq 3$ , variant frequency  $\geq 0.01$ , base quality  $\geq 10$ , and mapping quality  $\geq 20$ . Indel realignment was performed to correct underestimated allele frequencies. Variants were annotated using several databases containing functional (Ensembl, CCDS, RefSeq, Pfam), populational (dbSNP, 1000 Genomes, ESP6500, ExAC) and cancer-related information (COSMIC – Release 87, ICGC – Release 27), as well as nine scores from algorithms for prediction of the impact caused by nonsynonymous variants on the structure and function of the protein (SIFT<sup>31</sup>, PROVEAN<sup>32</sup>, Mutation Assessor<sup>33</sup>, Mutation Taster<sup>34</sup>, LRT<sup>35</sup>, MetaLR, MetaSVM<sup>36</sup>, FATHMM and FATHMM-MKL<sup>37</sup>), and one score (GERP++) for evolutionary conservation of the affected nucleotide.<sup>38</sup> Only variants described in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database were selected and filtered considering the following parameters: only variants with protein effect (codon stop, splicing, frameshift, nonsynonymous and insertions/deletions), variants with a minimum coverage of 20 reads and 3 reads with the alteration, *minor* allelic frequency (MAF)  $< 0.01$  (1%), minimum variant allelic frequency (VAF) cutoff of 0.05 (5%) and maximum

VAF cutoff of 0.35 (35%) to exclude possible germinal polymorphisms. The workflow performs a subsequent analysis to filter out recurrent sequencing errors, which is based on documented sequencing biases associated to NGS platforms.<sup>39,40</sup> All final variants were visually inspected in Integrated Genome Viewer.<sup>41</sup>

**Cytogenetic characterization.** Fluorescence in-situ hybridization (FISH) was performed at diagnosis on immunomagnetically enriched CD138+ plasma cells from 789/1,252 patients for IGH translocations, del(13q14), and del(17p13). Patients with t(4;14), t(14;16), and/or del(17p13) were classified as high-risk (N = 154); others were classified as standard-risk disease (N = 618).

**Statistical analyses.** The Mann-Whitney U non-parametric test was used to evaluate the statistical significance of differences observed between patients with or without MDS-PA. Survival probabilities according to the presence versus absence of MDS-PA at diagnosis were estimated using the Kaplan-Meier method, and compared with the use of a two-sided stratified log-rank test. The effect of MDS-PA on the risk of progression-free (PFS) and overall survival (OS) (hazard ratio; HR), with its two-sided 95% confidence interval (CI), were estimated with a logistic Cox regression model. PFS was defined as the time from MDS-PA assessment until disease progression or death from any cause, and OS was defined as the time from MDS-PA assessment at diagnosis until death. A multivariate Cox proportional hazard model was developed to explore the independent effect on PFS and OS of prognostic factors defining the revised international staging system (ISS): ISS, LDH levels and FISH cytogenetics. Statistical analyses were conducted using SAS (Institute Inc, Cary, NC), Stata (version 15.0; StataCorp LP, College Station, TX) and SPSS (version 20.0; IBM, Chicago, IL).

## RESULTS

**Incidence of MDS-PA in patients with newly-diagnosed MM.** Prospective screening of MDS-PA in a series of 285 transplant-eligible patients with MM, unveiled that 33 (11.6%) had MDS-PA at diagnosis. Detailed immunophenotypic analysis per lineage showed that neutrophil-lineage cells were more frequently altered (22/285, 7.7%), as compared to nucleated red blood cells (-NRBCs- 10/285, 3.5%) and monocytic cells (7/285, 2.5%). Only 5 (1.8%) patients had two or more cell lineages with MDS-PA at diagnosis.

Presence of MDS-PA and clonal hematopoiesis. To evaluate the correlation between the presence of MDS-PA and clonal hematopoiesis, we performed NGS of recurrently mutated genes in MDS in a total of 95 cell-population samples isolated from 67 patients at diagnosis. In cases without MDS-PA (N = 41) only CD34+ HPCs were selected, whereas in patients with MDS-PA (N = 26) CD34+ HPCs as well as the corresponding cell lineages displaying MDS-PA were sorted (Figure 1A). Overall, 31 mutations were detected being *TET2* the most frequently mutated gene (N = 8 cell-type/samples) followed by *NRAS* (N = 4) (Figure 1B). Approximately one-fifth (9/41, 22%) of cases without MDS-PA had detectable mutations in CD34+ HPCs; by contrast, half (13/26, 50%) of patients with MDS-PA displayed variants in CD34+ HPCs and/or maturing cell lineages (Figure 1C and Supplemental Table 4). Among patients with MDS-PA, most variants were detectable in CD34+ HPCs and less frequently in maturing cell lineages (Supplemental Table 4); however, in some cases, there were variants present in both CD34+ HPCs and maturing cells (Figure 1D). All mutations were sub-clonal with a median VAF of 8% (range, 5% - 27%), being higher in patients with vs without MDS-PA (9% vs 7%, respectively;  $P = .04$ ) (Figure 1E). The sub-clonal nature of mutations found in CD34+ HPCs and maturing cells was confirmed by scDNAseq (Figure 1F).

**Dynamics of MDS-PA and clonal hematopoiesis before and after autologous transplant.** In 84 of the 285 patients screened for MDS-PA at diagnosis, a subsequent analysis was performed at day 100 after HDT/ASCT in BM samples available after MRD assessment. In cases without MDS-PA at diagnosis (N = 68), 57 had no alterations after HDT/ASCT whereas in 11 cases, MDS-PA became detectable. Similarly, in 12/16 cases with MDS-PA at diagnosis (N = 16), these persisted after HDT/ASCT whereas in the remaining 4 patients, MDS-PA became undetectable (Figure 2A). Afterwards, we investigated in 18 patients whether those mutations profiles detected at diagnosis persisted after stem cell mobilization and ASCT, as well as if new variants were emerging after HDT. Thus, CD34+ HPCs were selected in 7

patients without MDS-PA at day 100 after HDT/ASCT, whereas CD34+ HPCs and respective lineages displaying MDS-PA were isolated in 11 cases with MDS-PA; all with NGS performed at diagnosis (Supplemental Table 5). Only two of the 18 patients displayed variants exclusively after HDT/ASCT, whereas in five cases either the same or different mutations were detected in both time points. By contrast, in 11 of the 18 cases, variants detected at diagnosis became undetectable after HDT/ASCT ( $N = 4$ ) or were never detectable at both time points ( $N = 7$ ) (Supplemental Table 5). Overall, both immunophenotypic and NGS analyses suggest that patients' phenotype is mostly driven by the findings at diagnosis, and that MDS-associated mutations unfrequently emerge after HDT/ASCT (Figure 2B).

**Impact of an altered hematopoiesis on the tumor microenvironment.** Based on PCA of the MFC antibody combination described above for screening of MDS-PA, a total of 14 cell clusters were identified and quantified at diagnosis and after HDT/ASCT (Figure 3A). Interestingly, patients with MDS-PA displayed recurrent alterations in the granulocytic differentiation when compared to cases without MDS-PA, with trends toward expanded CD34+CD117+ myeloid/erythroid precursors within HPCs (medians of 78% vs 72%, respectively;  $P = .05$ ), increased myelocytes (medians of 17% vs 11%, respectively;  $P = .04$ ) and decreased metamyelocytes (medians of 27,82% vs 14,89%, respectively;  $P = .003$ ) within total neutrophils (Figure 3B-E). Deep characterization of patients' T cells (14 cell clusters; Figure 4A) showed differences in the regeneration of the T cell compartment after stem cell transplantation. Namely, patients with MDS-PA showed reduced median frequencies of  $\gamma\delta$  naive T cells within total  $\gamma\delta$  T cells (5% vs 23%, respectively;  $P = .008$ ) (Figure 4B) as well as central memory CD4 (25% vs 38%, respectively;  $P = .02$ ) and CD8 (1% vs 6%, respectively;  $P = .025$ ) T cells (Figure 4C), together with an expansion of CCR7 negative Tregs at the expense of the CCR7 positive subset (64% vs 42%, respectively;  $P = .001$ ) (Figure 4D) as compared to cases without MDS-PA.

**Clinical significance of an altered hematopoiesis.** After unveiling that MDS-PA was often associated with clonal hematopoiesis and impacted on the tumor microenvironment, we sought to investigate if such abnormalities had clinical relevance. Accordingly, we analyzed the significance of CD56+ aberrant monocytes since data on this MDS-PA was available in a large series of 1252 transplant-eligible and ineligible patients, enrolled in four PETHEMA/GEM protocols. Overall, 70 of the 1252 (5.6%) cases displayed this MDS-PA at diagnosis (Table 1). Of note, this frequency was higher to that found in the GEM2012MENOS65 trial (2.5%) since it was

significantly ( $P = .005$ ) increased in elderly, transplant-ineligible patients enrolled in the GEM2005MAS65 study (9.7%), as well as in cases included in the GEM2000 protocol (6.1%) which enrolled up to the age of 70 (Table 1). Thus, we found a significant correlation between the presence of MDS-PA and increased age (65 vs 60 years in cases without MDS-PA;  $P = .04$ ), but also with lower hemoglobin values (97 vs 106 g/dL;  $P = .04$ ), high LDH levels (21% vs 13%,  $P = .04$ ), higher BM PC infiltration (54% vs 35%,  $P = .002$ ) and higher incidence of ISS III (40% vs 24%,  $P = .004$ ) at diagnosis. In addition, patients with MDS-PA showed more frequently anemia (80% vs 55% in cases without MDS-PA,  $P = .002$ ) and neutropenia (70% vs 50%,  $P = .01$ ) during treatment. No differences were observed regarding the incidence of high-risk cytogenetic abnormalities. Patients with MDS-PA had significantly inferior PFS and OS when compared to cases without MDS-PA (median PFS of 26 vs 42 months,  $P = .001$  and median OS of 50 vs 79 months,  $P = .004$ , respectively) (Figure 5). Furthermore, the presence of MDS-PA had independent prognostic value for PFS (HR: 1.5, 95% CI [1.1 – 2.1];  $P = .02$ ) and OS (HR: 1.7, 95% CI [1.1 – 2.5];  $P = .01$ ) in a multivariate analysis together with other adverse parameters such as ISS III, elevated LDH and high-risk FISH abnormalities (Table 2). Most interestingly, the negative impact of MDS-PA in survival was solely observed in patients receiving maintenance without IMiDs, and it was abrogated whenever thalidomide or lenalidomide were used (Supplemental Figure 3). That notwithstanding, presence of MDS-PA retained independent prognostic value for PFS (though not for OS) in a multivariate model including the use of IMiDs for maintenance as a variable (Supplemental Table 6).

## DISCUSSION

Unexplained blood cytopenias can be a clinical challenge and the relationship between these and somatic mutations in genes known to be associated with MDS (ie. CHIP and clonal cytopenias of undetermined significance [CCUS]) is an area of extensive research. Of note, up to 30% of patients with non-Hodgkin lymphoma have detectable CHIP and this is associated with inferior survival.<sup>42</sup> Here, we conducted the largest study reported thus far on the incidence and significance of dysplastic hematopoiesis in untreated MM and found that approximately 1 out of 10 patients display MDS-PA at diagnosis. Moreover, targeted sequencing of MDS recurrently mutated genes in CD34+ progenitors (and dysplastic lineages) unveiled clonal hematopoiesis in half the cases with MDS-PA; *TET2* and *NRAS* being the most frequently mutated genes (Supplemental Table 7). These findings had important clinical translation since the presence of MDS-PA at diagnosis anticipated greater risk of hematological toxicity and was independently associated with inferior survival.

Cytopenias may induce treatment-related complications leading to dose reduction or greater time intervals. More than 10 years ago, Barlogie and colleagues reported that MDS-CA after HDT/ASCT was associated with lower CD34+ HPC' yield at collection, longer time interval from MM diagnosis to HDT, older age, and lower platelet recovery after HDT.<sup>10</sup> Chitre et al have recently shown that most MM patients with detectable CHIP in leukapheresis prior to HDT/ASCT were anemic and one case had neutropenia, although no association between CHIP and cell numbers at apheresis was observed.<sup>12</sup> Here, we confirmed and expanded these findings by showing a similar association between an immunophenotypically altered hematopoiesis and anemia at diagnosis, together with anemia and neutropenia during treatment. We found no correlation between MDS-PA at diagnosis and impaired mobilization of CD34+ HPCs (data not shown).

We and others have reported that the composition and functionality of CD34+ HSCs is dysregulated in patients with active MM, possibly due to a progressively increasing competition between tumor PCs and hematopoietic progenitors for potentially overlapping (SDF-1 related) BM niches and activated TGFβ signaling in the BM tumor microenvironment.<sup>43,44</sup> Here, our results suggest that presence of dysplastic hematopoiesis with underlying somatic mutations at the stem cell level could also contribute to the high frequency of anemia at diagnosis and during therapy. However, it should be noted that due to the small number of patients with paired phenotypic and genetic analyses, this study is not powered to establish a relationship between MDS-PA and clonal hematopoiesis.

To date, whether HDT/ASCT favors the emergence of dysplastic hematopoiesis remains controversial. Based on longitudinal evaluation of MDS-PA at diagnosis and after autologous transplant, we describe here that in most cases (80%), MDS-PA either persisted or remained absent in patients with or without MDS-PA at diagnosis, and that MDS-associated mutations unfrequently emerged after high-dose therapy. Overall, both immunophenotypic and NGS analyses suggest that patients' phenotype is mostly driven by the findings at diagnosis, but these data should be interpreted with caution due to the sub-clonal nature of detected mutations (e.g. a mutation appearing after HDT/ASCT could have been present in a minor sub-clone at diagnosis). Notwithstanding, screening immunophenotypic features of dysplasia could be an informative test in patients where the degree of cytopenia is not explained by the myeloma tumor load or treatment being used.<sup>45</sup> Interestingly, none of the patients with MDS-PA had evidence of morphologic dysplasia after cytological assessment of BM smears.

The BM milieu is a crucial modulator of the MM biology/pathogenesis.<sup>46–54</sup> Accordingly, patients with MM are highly susceptible to infections, owing to diminished innate and adaptive immune repertoires and function.<sup>55,56</sup> Immune dysfunction may occur either as a consequence of an altered microenvironment due to progressive expansion of tumor PCs, or secondary to therapy.<sup>57</sup> However, the contribution of dysplasia to both altered hematopoietic and immune profiles in patients with MM has not been investigated. Here, we unravel that similarly to many cases with MDS, newly-diagnosed MM patients with MDS-PA show an expansion of myeloid/erythroid precursors at expenses of lymphoid precursors within the CD34+ HPC compartment, and a maturation arrest in immature neutrophil stages.<sup>58,59</sup> Of note, altered distribution of  $\gamma\delta$  T cells and Tregs have been reported in MDS<sup>60,61</sup> and here, we found that MM patients with MDS-PA showed significantly reduced frequencies of  $\gamma\delta$  naive T cells as well as a significant expansion of CCR7 negative Tregs at expenses of the CCR7 positive subset (when compared to cases without MDS-PA). Thus, further research is warranted to explore if these findings result in impaired cell function, as well as the clinical relevance of immune alterations resulting from dysplastic hematopoiesis and if this can be abrogated with specific immunotherapies. Of note, in 47 patients with longitudinal evaluation of MDS-PA from diagnosis onto maintenance, MDS-PA identified at diagnosis became undetectable during maintenance in 3 of 5 patients; by contrast, among 42 cases without MDS-PA at diagnosis, 12 displayed MDS-PA during maintenance.

In line with previous observations based on MDS-CA or CHIP<sup>10,12,62</sup>, we found no statistically significant association between presence of MDS-PA and development

of MDS or AML after a median follow-up of 5 years (range, <1 - 15), probably due to its generally low incidence and the long-time between diagnosis of MM and subsequent diagnosis of MDS/AML.<sup>5,11</sup> However, we and others have shown that MDS or AML are often preceded by detectable MDS-PA or MDS-CA<sup>10,11,14</sup>, which suggests that their presence could be useful to monitor closely for these two SPM. Furthermore, we confirmed previous observations made by Barlogie et al<sup>10</sup> that patients with dysplastic hematopoiesis have inferior survival and unveiled that IMiD-based maintenance may overcome its negative prognostic value. Taken together, these studies support cost-effective screening of dysplasia in patients with PC disorders to help resolving unexplained cytopenias and to identify treatment strategies overcoming its clinical consequences for improving patients' quality of life. Noteworthy, the results from another study analyzing the presence of CHIP in growth factor-mobilized peripheral blood from a large series of transplant-eligible patients, reproduced our findings in such a way that the use of IMiD's post ASCT abrogated the deleterious effects of CHIP on PFS and OS.<sup>62</sup> Thus, the results from both studies support the use of IMiD's during maintenance in patients with MDS-PA (or CHIP) at diagnosis.



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## AUTHORSHIP CONTRIBUTIONS

B.P. and A. Orfao conceived the idea and B.P. designed the study protocol. C.M., N.P., MT.C., JJ.P., J.M., C.M., and L.B. analyzed flow cytometry data. D.A. performed cell sorting. I.V., P.A, S.S. and MC.C. performed next-generation sequencing; C.M., R.V-M., I.V., MC.C. F.J. G-A, G.A and I.G. analyzed sequencing data. MJ.C., J.M.L., F.J.G-A., G.A., R.GS., M.G., C.B., F.P., S.M., A.Orfao., A.Oriol, Al.T., MA.E., R.d.P, F.dA., M.T.H., L.P., R.M., L.R., MV.M, JJ.L., J.B., and J.F.S.M. provided study material and/or patients. C.M. and B.P. performed statistical analysis. C.M., J.F.S.M and B.P. wrote the manuscript. All authors reviewed and approved the manuscript.

## CONFLICTS OF INTEREST

N.M. reports consultancy and honoraria from Amgen, research funding, honoraria, membership on an entity's Board of Directors or advisory committees, speakers bureau and consultancy from Celgene, research funding, consultancy and honoraria from Janssen, honoraria and consultancy from Takeda and honoraria from The Binding site. J.M.L advisory committee, honoraria or research support from Novartis, Celgene, Janssen, BMS, Incyte and VIVIA. A. Oriol participated in advisory boards for Amgen, Celgene and Janssen. F.d.A. reports honoraria for lectures from Celgene, Janssen, Amgen and Takeda, and consultancy with Amgen, Celgene and Janssen. M.-V.M. has received honoraria from lectures or participation in advisory boards from Janssen, Celgene, Amgen, Takeda, Abbvie, Adaptive, GSK, Pharmamar, EDO, Oncopeptides. L.R. reports honoraria from Janssen, Celgene, Amgen, and Takeda. J.B. reports honoraria for lectures from Janssen, Amgen, Celgene, Takeda and Oncopeptides. J.-

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**Table 1.** Patient characteristics at diagnosis plus frequency and type of hematological toxicity during treatment.

Diagnostic features	No MDS-PA (n=1182; 94,4%)	MDS-PA (n= 70; 5,6%)	P - value
Age (years)*	60	65	.04
Sex (male)	659 (53%)	38 (54%)	.86
Hemoglobin (g/dL)*	106	97	<.001
LDH (elevated)	154 (13%)	15 (21.4%)	.04
ISS (n= 1211)	n=1143	n=68	
I	405 (35,4%)	14 (20,6%)	.007
II	462 (40,4%)	27 (39,7%)	.507
III	276 (24,2%)	27 (39,7%)	.004
Isotype (n=1239)	n= 1171	n=68	
IgG	689 (58,8%)	32 (47%)	.038
IgA	291 (24,9%)	19 (27,9%)	.328
IgD	9 (0,77%)	1 (2,1%)	.433
IgM	1 (0,09%)	-	-
Light-chain	181 (15,5%)	16 (23%)	.060
Plasma cells (%)*	35	54	.002
Cytogenetics (n=772)	n=726	n=46	
Standard-risk	581 (80%)	37 (80%)	.562
High-risk <sup>#</sup>	145 (20%)	9 (20%)	.562
<b>Hematological toxicity*</b>	<b>No MDS-PA (N = 736; 94.8%)</b>	<b>MDS-PA (n= 40; 5.3%)</b>	<b>P - value</b>
Anemia	405 (55%)	32 (80%)	.002
Neutropenia	364 (50%)	28 (70%)	.011
Thrombocytopenia	195 (27%)	11 (28%)	.889

\* Median values

<sup>#</sup> t(4;14), t(14;16) and/or del(17p)

¥ Data on hematological toxicity during treatment was unavailable in patients enrolled in the GEM2000 protocol

**Table 2.** Multivariate analyses of prognostic factors for progression-free survival (PFS) and overall survival (OS) in patients with newly diagnosed multiple myeloma (N = 1252), including the presence of MDS-PA based on aberrant CD56 expression in the monocytic lineage.

	PFS		OS	
	HR (95% CI)	P	HR (95% CI)	P
<b>ISS stage III</b>	1.3 (1.0 – 1.6)	.029	1.7 (1.3 – 2.1)	.000x10 <sup>4</sup>
<b>Elevated LDH levels</b>	1.7 (1.3 – 2.2)	.00	1.9 (1.4 – 2.6)	.000x10 <sup>5</sup>
<b>t(4;14), t(14;16), del(17p)</b>	1.3 (1.1 – 1.6)	.009	1.5 (1.2 – 1.9)	.000x10 <sup>4</sup>
<b>MDS-PA</b>	1.5 (1.1 – 2.1)	.016	1.7 (1.1 – 2.5)	.012

HR: hazard ratio; CI: confidence interval; ISS: International staging system; LDH: Lactate dehydrogenase; MDS-PA: myelodysplastic-like phenotypic abnormalities

**FIGURE LEGENDS**

**Figure 1. MDS-associated variants in patients with newly-diagnosed MM. (A)** Study design. **(B).** Frequency of mutations identified in a total of 95 cell-types/samples from 67 patients: 41 without MDS-associated phenotypic abnormalities (MDS-PA) in whom CD34+ hematopoietic progenitor cells (HPCs) were selected, and 26 with MDS-PA in whom CD34+ HPCs and respective lineages displaying MDS-PA were isolated. Mutations were broken down according to the type of variant. **(C)** Frequency of detectable mutations in patients with or without MDS-PA. **(D)** Patients with mutations present in both CD34+ HPCs and mature dysplastic cells (#746, #810). NRBCs: nucleated red blood cells. **(E)** Median variant allele frequency (VAF) in patients with or without MDS-PA **(F)** Mutations detected by single-cells DNA sequencing in CD34+ HPCs and/or dysplastic myeloid lineages from four patients with MDS-PA. \* $P \leq 0.05$

**Figure 2. Dynamics of MDS-PA and clonal hematopoiesis before and after autologous transplant. (A)** Longitudinal screening of MDS-associated phenotypic abnormalities (MDS-PA) in 84 patients, at diagnosis and at day 100 after high-dose therapy followed by autologous stem cell transplantation (HDT/ASCT). **(B)** Distribution of patients (N = 18) according to the detection of mutations in both time points.

**Figure 3. Altered hematopoiesis in patients with MDS-PA. (A)** Identification of various immature and mature cell types in the tumor microenvironment of a representative patient, using multidimensional flow cytometry and principal component analysis (PCA): CD34+CD117+ myeloid/erythroid and CD34+CD117- lymphoid precursors within CD34+ hematopoietic progenitor cells (HPCs), mesenchymal stem cells, basophils, eosinophils, mast cells, monocytes, four neutrophil subsets (promyelocyte, myelocyte, metamyelocyte and band/mature neutrophils), two nucleated red blood cell-subsets (NRBCs; proerythroblasts and erythroblasts), and lymphocytes. **(B)** Percentage of CD34+CD117+ myeloid/erythroid precursors within CD34+ HPCs in patients with (N = 33) or without MDS-PA (N = 243) at diagnosis **(C)** Immunophenotypic characterization of neutrophil maturation dissected into the promyelocytic, myelocytic, metamyelocytic and band/mature neutrophil stages based on differential expression of CD11b and CD13. **(D)** Percentage of myelocytes within the neutrophil compartment in patients with (N = 22) or without (N = 62) MDS-PA after HDT/ASCT. \* $P \leq 0.05$ . **(E)** Percentage of metamyelocytes within the neutrophil compartment in patients with (N = 19) or without (N = 40) MDS-PA at diagnosis. \* $P \leq 0.05$ .

**Figure 4. Patients with MDS-PA display altered T cell regeneration after autologous transplant.** (A) Identification of various T cell subsets using multidimensional flow cytometry and principal component analysis (PCA) in patients with (N = 9) or without MDS-PA (N = 33): naïve, central memory, effector memory, transitional memory and terminally differentiated CD4 T cells, CCR7- and CCR7+ T regulatory cells (Tregs), naïve, central memory, effector memory, effector and terminally differentiated CD8 T cells, as well as naïve and effector memory TCR $\gamma\delta$  T cells. Percentages of (B)  $\gamma\delta$  naïve T cells within total  $\gamma\delta$  T cells, (C) CD4 and CD8 central memory within their respective CD4 and CD8 compartments and (D) CCR7 negative and positive subsets within T regulatory cells. \* $P \leq .05$  and \*\* $P \leq .01$ .

**Figure 5. Survival according to undetectable versus detectable MDS-PA.** The Kaplan–Meier estimates of progression-free and overall survival after the assessment of MDS-associated phenotypic abnormalities (MDS-PA) in the monocytic lineage at diagnosis (N =1252) are shown in A and B. HR: hazard ratio; CI: confidence interval.

**Figure 1.**

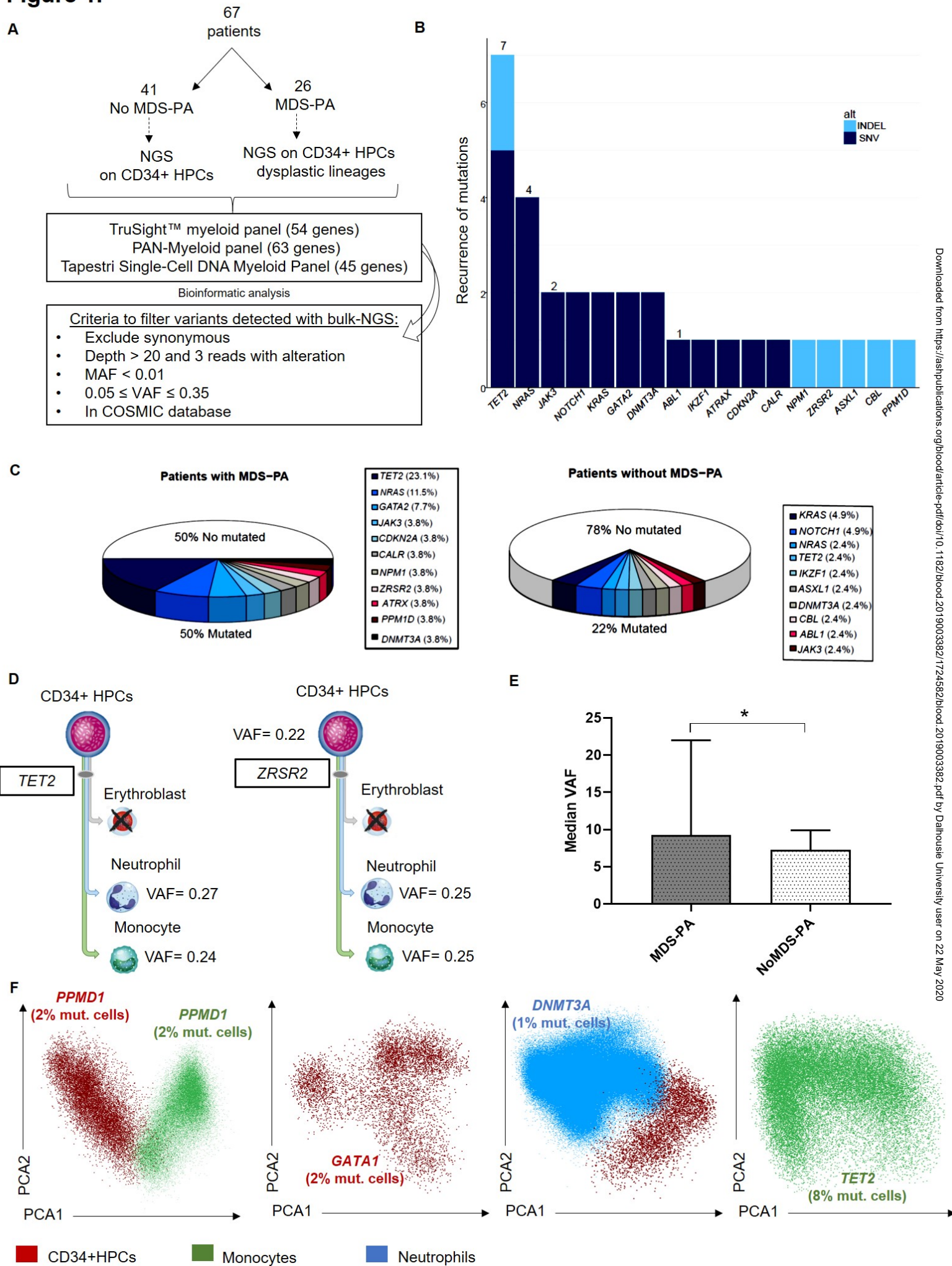
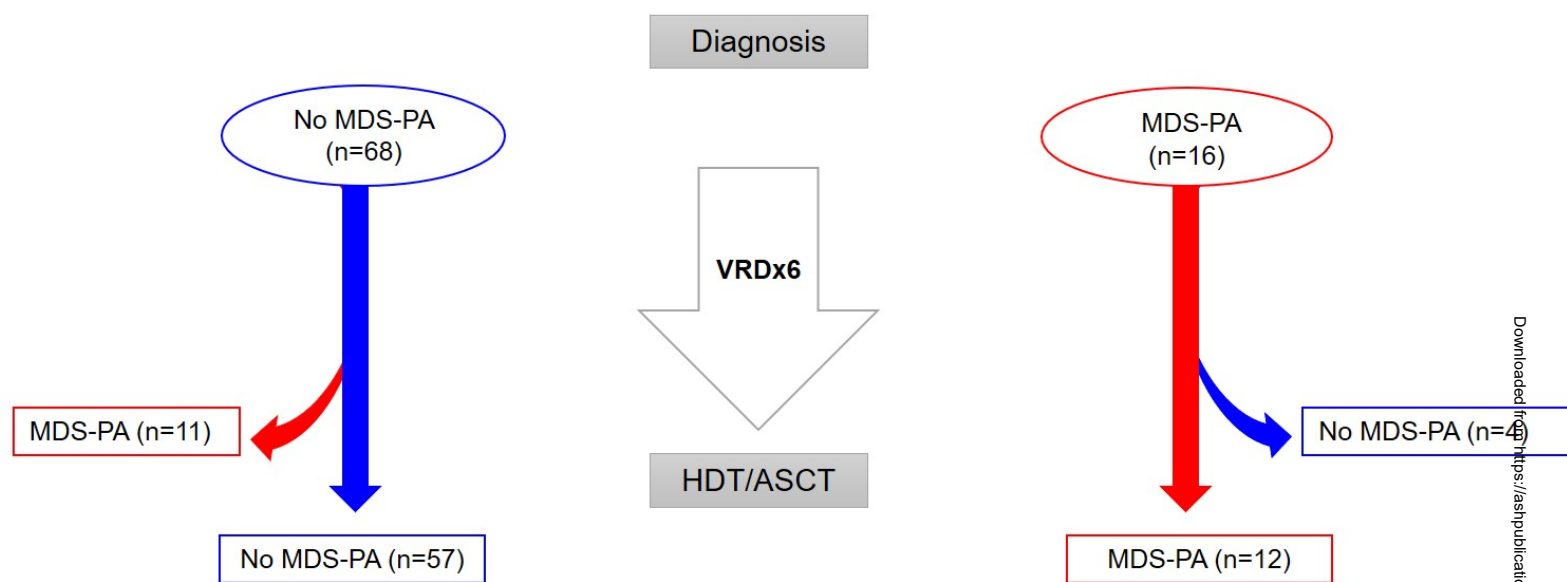


Figure 2.

A



B

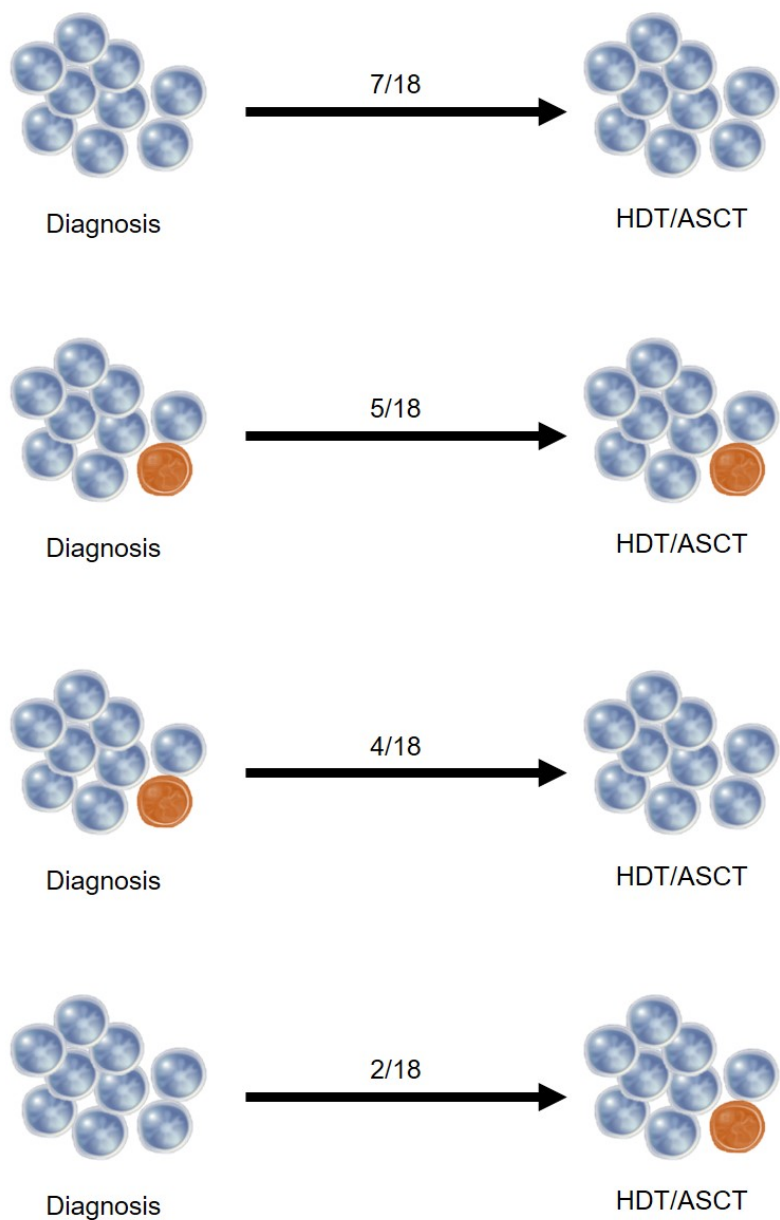
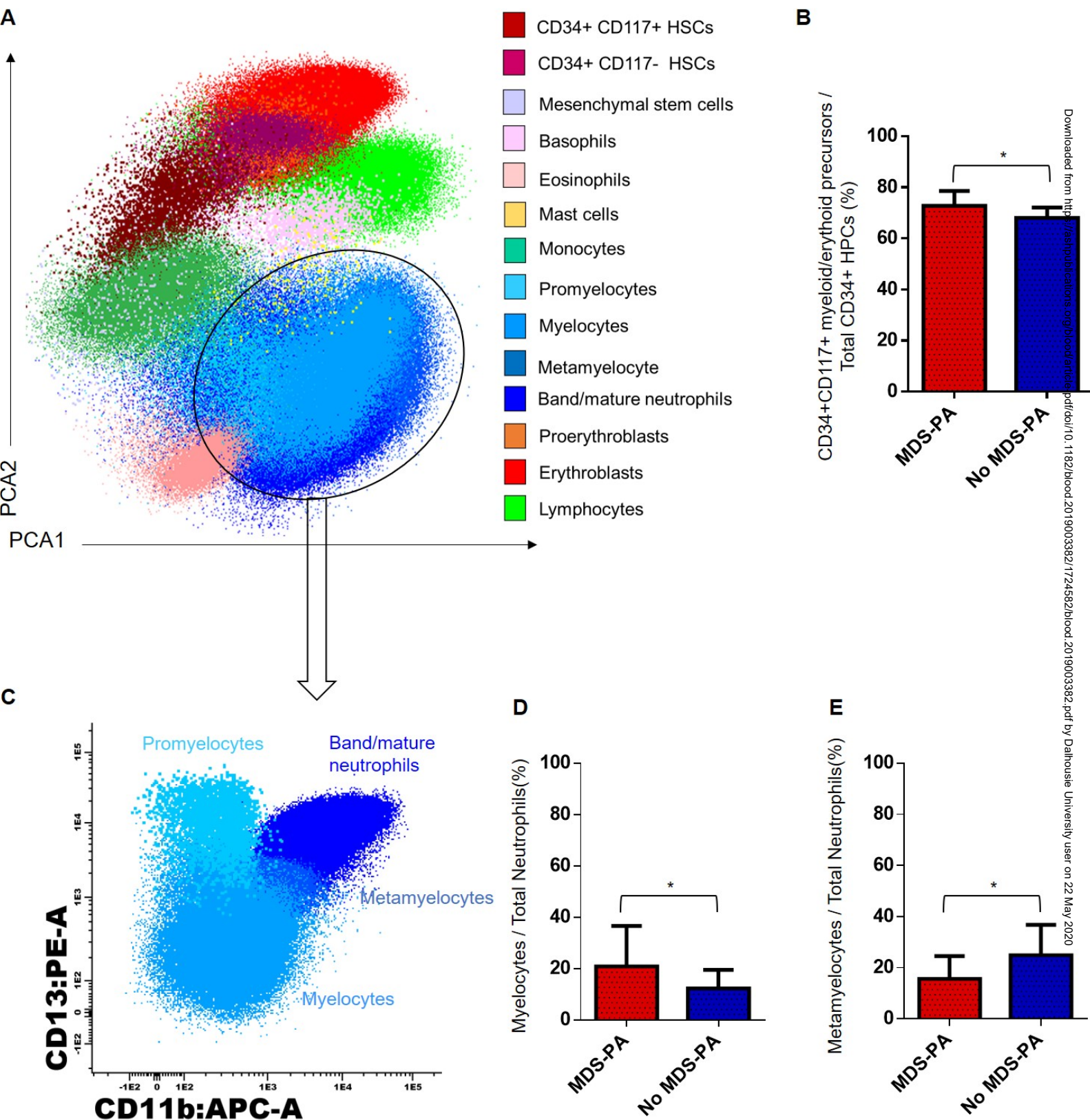


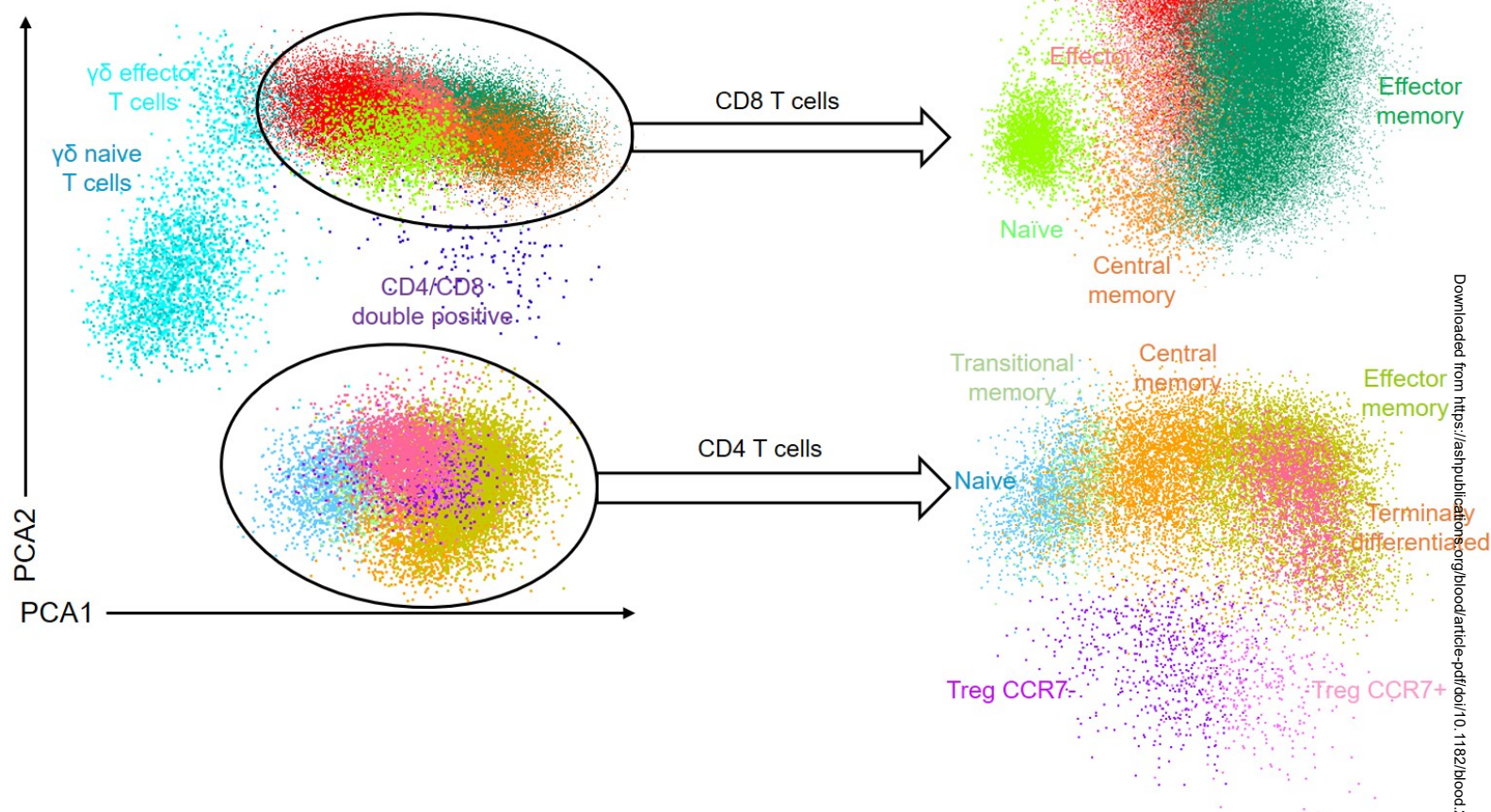


Figure 3.



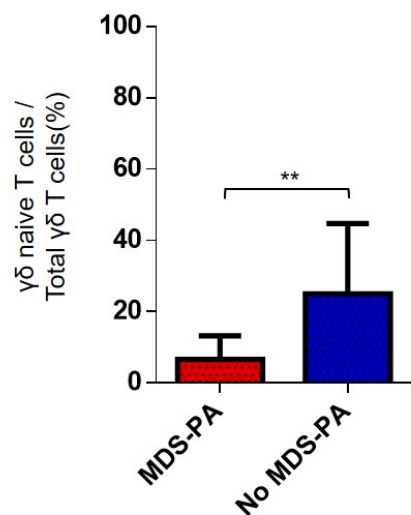
**Figure 4.**

**A**

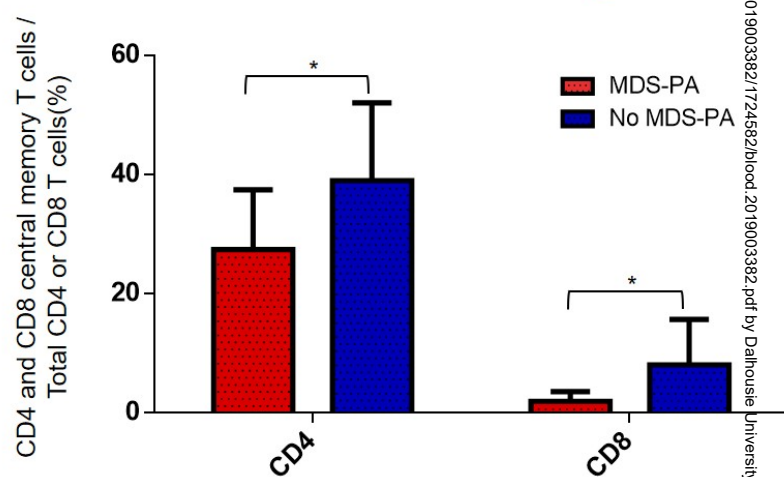


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**B**



**C**



**D**

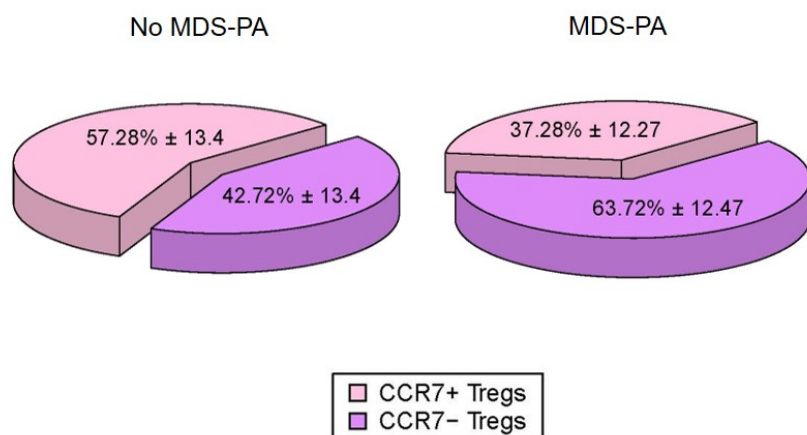
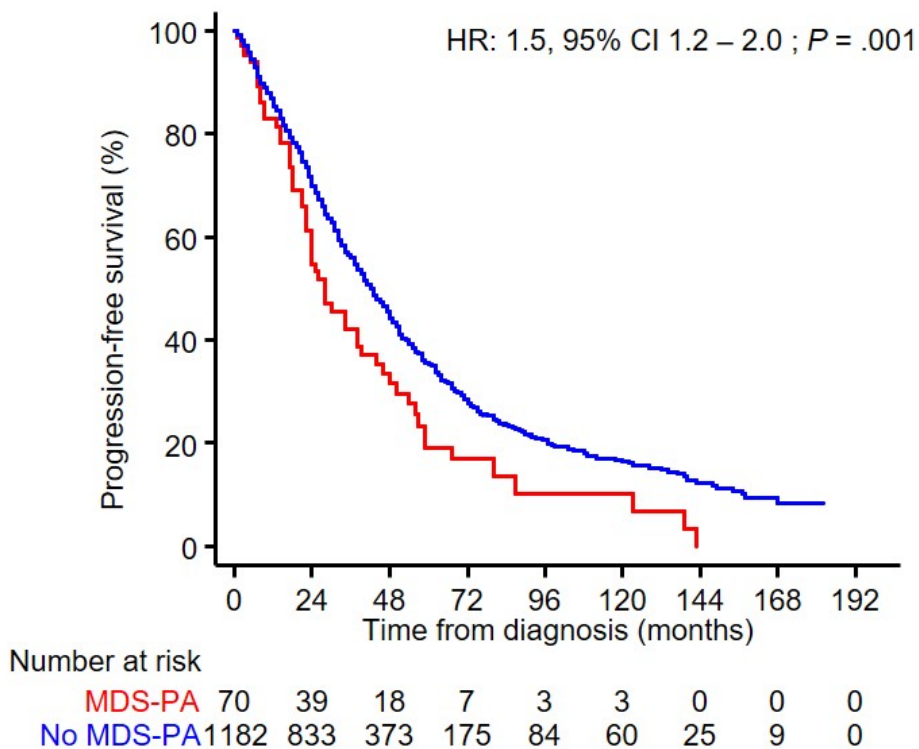




Figure 5.

A



B

