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Abstract

Multiple myeloma (MM) is an incurable neoplastic plasma cell (PC) disorder characterized by proliferation of clonal/aberrant PCs in bone marrow, usually preceded by a premalignant stage known as monoclonal gammopathy of undetermined significance (MGUS). Diagnosis of MGUS and MM, and minimal residual disease assessment rely on multiparameter flow cytometry (MFC), which allows immunophenotypic characterization of clonal vs normal/reactive PCs, elaboration of prognostic considerations and study of the other cellular populations of the sample. Some critical points remain to be solved: i) exact definition of normal PC immunophenotype; ii) evaluation of relationship between depth of response/presence of relapsing disease and both clonal and normal PC immunophenotype; iii) introduction of new PC detection markers; iv) wider understanding of connection among relapse/response-PC immunophenotype-alterations in bone marrow microenvironment. In my PhD research I provided some new insights in these directions. My original contribution to knowledge is the dissection of the relation existing between the influence that malignancy exerts on other cells, including normal PCs, and the characteristics of the disease, in terms of clonal PC immunophenotype and achieved response. I demonstrated that normal and clonal PCs are not equal among different categories of patients, so I proposed the adoption of response/relapse related MFC panels and the introduction of normal PC analysis to follow the various phases of disease. I recognized CD229 as a new identification marker suitable for test in clinical routine, especially in patients undergoing to therapies which may compromise efficient PC staining using classical CD38 and CD138 markers. Studying distribution of bone marrow naïve/transitional and memory B cells, I detected significant differences among stringent complete responders and other treated patients. Thus, I suggested a possible role of naïve/transitional and double negative B cells in prediction of depth of response. I explored the influence of CD117 clonal PC positivity on CD34+ hematopoietic progenitor cell (HPC) distribution, and I demonstrated that response to treatment takes place through different forms in CD117- and CD117+ subjects, involving a diverse partition of CD34+ HPCs into CD34+CD19- and CD34+CD19+ subsets, which becomes evident comparing untreated and treated CD117- patients, but is impossible to detect in CD117+ cases.

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1. Introduction

Multiple myeloma is an incurable neoplastic plasma cell disorder characterized by proliferation of clonal malignant plasma cells in bone marrow, and presence of monoclonal immunoglobulin (M-protein) in blood and/or urine, associated with immunodeficiency and related organ or tissue impairment (end-organ damage) usually manifested through CRAB signs (hypercalcemia, osteolytic bone lesions, renal insufficiency, anemia) [1-4]. Median age at diagnosis is between 65 and 70 years [4]. As indicated by Moreau et al. [4] the disease represents 1% of all cancers and ~10% of all haematological malignancies. The incidence in Europe is 4.5–6.0/100,000/year, with a mortality of 4.1/100,000/year [4]. Multiple myeloma is usually preceded by a premalignant plasma cell proliferative stage characterized by asymptomatic M-protein production known as monoclonal gammopathy of undetermined significance (MGUS) [3, 5]. MGUS has a prevalence of 3.2% in the white general population ≥50 years of age [5]; in some of these patients, MGUS progresses to smoldering (asymptomatic) multiple myeloma (SMM) and to multiple myeloma, with a risk of around 1% per year for patients with MGUS [2-3, 6].

Progression from MGUS to multiple myeloma is associated with a variety of genetic alterations and changes in the bone marrow microenvironment involving cytokines and growth factors as mediators, but also direct cellular interaction, influencing clonal plasma cell expansion, immune suppression, and augmented angiogenesis and bone resorption [2-3].

In the last years, the introduction of novel antimyeloma agents bortezomib (proteasome inhibitor), and thalidomide and lenalidomde (immunomodulatory drugs), flanking autologous stem cell transplantation (ASCT), has improved survival significantly [3, 7], both in post-ASCT relapse and in new diagnosis scenarios [8]. The adoption of these new approaches has produced a 50% improvement in median survival [8-9]. As revealed in a study by Brenner et al. [10] related to 2002-2004 period, the most prominent progresses were observed in younger patients (<50 years), leading to 5- and 10-year relative survival of 56.7% and 41.3%, and in subjects whose age is comprised between 50 and 59 years, with a 5- and 10-year relative survival of 48.2% and 28.6%. In turn, only modest improvement was achieved in patients with 60–69 years of age, and no significant amelioration was reached in older patients [10]. Despite these improvements in survival, the course of multiple myeloma is still characterized by a pattern of remission and relapse, with decrease in durability of response and increase in frequency of salvage therapies, and the final developing of a refractory stage. This pattern reflects the persistance of residual tumour cells even after ASCT or the reaching of a complete response to treatment [3, 11-13].

In multiple myeloma diagnosis, monitoring, transplant settling and elaboration of prognosis, clinicians may take advantage from many technical interventions, like protein and imaging studies, bone marrow morphologic evaluation, allele-specific oligonucleotide polymerase chain reaction (ASO-PCR), deep-sequencing and multiparameter flow cytometry (MFC) [2, 14-15]. Every cited approach clearly offers a panel of advantages, but only MFC allows a rapid enumeration and characterization of clonal plasma cells, providing immunophenotypic data which may be used for

minimal residual disease (MRD) assessment and prognostic purposes. Moreover, while molecular approaches may be centered on the detection of specific genetic markers in whole marrow populations, or associated with high technical complexity, and are time consuming and relatively expensive (and therefore less applicable in routine clinical practice), MFC permits to perform single cell focused studies, demonstrates a large applicability, requires shorter time to prepare/analyze the sample, is associated with good levels of sensitivity (higher than 10^{-4}-10^{-5} with new instruments and analysis of up to $2x10^{6}$ cells) and offers a high level of concordance in MRD detection with deep sequencing [14-15]. In addition, MFC renders possible simultaneous exploration of immunophenotype and characterization of other cellular populations present in the same sample. Nowadays, from a practical point of view, some aspects of myeloma management relies only on MFC in clinical routine, like for example assessment of immunophenotypic complete response in treated patients (as I discuss in the following paragraph). MFC was regarded as highly dependent on expertise and poorly reproducible in multicenter studies, mostly because of the lack of standardization in data analysis, interpretation, and presentation, and limited evaluation of "new" vs "classical" markers [16]. However many efforts have been made in order to ensure standardization of procedures and instrument settings [15, 17-18], and MFC has been proved to be considerably useful in diagnosis and prognostic stratification of multiple myeloma, as well as other hematological malignancies [15, 18]. In the following sections, I briefly discuss the state of art for multiple myeloma and related condition MGUS as regards diagnosis, definition of prognosis and monitoring, in order to provide the conceptual basis to critical interpret and to understand the research lines deepened in this thesis. For each aspect, I focus on the role of MFC and the most critical unsolved questions.

1.1 Defining the variants.

As indicated in WHO classification [2], ~97% of patients with multiple myeloma present an M-protein in the serum or urine (lgG 50%; IgA 20%; light chain 20%; IgD, IgE, IgM and biclonal < 10%). The remaining ~3% of cases are non-secretory subjects. In this last category, 85% of patients shows defects in immunoglobulin secretion, since cytoplasmic M-protein may be detected. In turn, 15% of patients are true non-producer myeloma cases. In up to two-thirds of non-secretory myeloma cases, increased serum free light chains and/or an abnormal free light chain (FLC) ratio may be detected [2]. As regards MGUS, 3 distinct clinical subtypes (non-IgM MGUS, IgM MGUS, and light-chain MGUS) have been distinguished [2, 19-20]. IgM MGUS tends to progress to other disease entities (such as Waldenström macroglobulinemia) and only sporadically to the extremely rare entity IgM multiple myeloma. Instead non-IgM MGUS and light chain MGUS are premalignant condition of non-IgM multiple myeloma and light chain multiple myeloma, respectively [2, 19-20]. In the following chapters, “multiple myeloma”, “MGUS” and “SMM” are used to indicate all the variants of the respective category without distinctions, unless otherwise specified.

1.2 Diagnosis, definition of prognosis and monitoring: still looking for the light.

Each aspect of diagnosis and management of MGUS and multiple myeloma are described synthetically in specific paragraphs. A more profound dissertation about these topics may be found in bibliographical references reported in the paragraphs.

1.2.1 Diagnosis.

Diagnosis of MGUS and multiple myeloma is a multistep process resorting to clinical chemical, serological and immunological laboratory analysis, morphological evaluation, imaging, cytogenetic and MFC investigation. As MGUS is an asymptomatic condition, a crucial parameter for a differential diagnosis vs multiple myeloma is the absence of end organ damage, mostly defined as CRAB signs [19-23].

CRAB stands for hyper*c*alcemia (serum calcium ≥11.5 mg/dL), *r*enal insufficiency (serum creatinine >2 mg/dL), *a*nemia (normochromic, normocytic with a hemoglobin >2 g/dL below the lower limit or hemoglobin <10 g/dL) and *b*one lesions (lytic lesions or osteoporosis with pathologic fractures) [2, 20, 22-25]. Other recognized signs of organ or tissue impairment are symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections (>2 episodes in 12 months) [20, 24-25]. Specifically in case of IgM MGUS, criteria are slightly different, and include no evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly that can be attributed to the underlying disorder [19, 21-22]. To complete a diagnosis of MGUS, serum M-protein must be <3 g/dL, and clonal bone marrow plasma cells (or lymphoplasmacytic cells in case of IgM MGUS) must be <10%. In case of light chain MGUS, criteria for percentage of bone marrow plasma cells and absence of end organ damage do not change. However, some additional requirements are necessary: abnormal FLC ratio (<0.26 or >1.65), increased level of the appropriate involved light chain (increased kappa FLC -in subjects with ratio >1.65- and increased lambda FLC -in patients with ratio<0.26-), and no immunoglobulin heavy chain detection on immunofixation [19-22]. As reported by Engelhardt et al. [26], MGUS with renal impairment (RI) has been recognized as an independent entity and thus called 'monoclonal gammopathy of renal significance' (MGRS) [26]. SMM is also an asymptomatic condition. It may be distinguished from MGUS because it requires serum M-protein ≥3 g/dL and/or clonal bone marrow plasma cells ≥10%. Presence of end organ damage, with clonal bone marrow plasma cells ≥10% or biopsy proven plasmacytoma (i.e. masses of monoclonal plasma cells), and presence of serum and/or urinary M-protein (except in patients with true nonsecretory disease) are criteria to set a diagnosis of multiple myeloma [2, 19-20, 22-25]. Even in absence of end organ damage, presence of 60% of clonal plasma cells is a valid requisite to establish a diagnosis of multiple myeloma [21]. In IgM multiple myeloma, diagnostic criteria include clonal bone marrow plasma cells ≥10%, detection of serum IgM monoclonal protein, and presence of lytic bone lesions and/or translocation t(11;14) detected on fluorescence in situ hybridization [19-20].

Other clinical manifestations of multiple myeloma include appearance of focal tumoural masses of plasma cells. Extramedullary manifestations generally develop in advanced phases of the disease. In turn, plasma cell leukemia (in which the number of clonal plasma cells in peripheral blood is > $2x10^9$/L or is 20% of the leukocyte differential count) may be present at the time of diagnosis (primary) or appear lately (secondary) [2].

1.2.2 Definition of prognosis and its influence on the choice of therapy.

MGUS patients are generally not treated, but only monitored in order to detect progression of disease [26]. The risk of progression is estimated on the basis of two models as indicated in Korde et al. [20]. The Mayo Clinic risk stratification model recognizes as risk factors non-IgG isotype, serum M-protein levels >1.5 g/dL and abnormal FLC ratio (<0.26 or >1.65) [20]. Instead the so called Spanish study group has specified as risk factors MFC aberrant plasma cell/total bone marrow plasma cell ratio (aberrant plasma cells within the bone marrow plasmacellular compartment) ≥95% and DNA aneuploidy (further data are provided in the following paragraphs) [20]. Also for SMM patients risk progression factors were determined. The Mayo Clinic risk stratification scheme uses bone marrow plasma cells ≥10%, serum M-protein levels ≥3 g/dL and abnormal FLC (<0.125 or >8) to evaluate the risk of progression [20]. In turn, the Spanish study group reported aberrant plasma cell/total bone marrow plasma cell ratio ≥95% and immunoparesis as risk factors for progression [20]. As regards treatment of SMM cases, conflicting reports has not brought to a final agreement; following the Mayo Clinic updates included in the Mayo Stratification for Myeloma and Risk-adapted Therapy (mSMART) reports [21,27], treatment may be indicated exclusively in a small subset of subjects considered at risk of imminent progression (including patients with serum FLC ratio ≥100), although the ESMO (European Society for Medical Oncology) Clinical Practice Guidelines and the European Myeloma Network recommendations do not adopt automatically these criteria [4, 21, 26-27]. Symptomatic myeloma patients are always treated, though the type of treatment depends on a series of factors. There is an unsolved “cure versus control” debate about whether myeloma should be treated with an aggressive multi-drug strategy with the objective of achieving complete response, or through a sequential disease control approach that puts the accent on quality of life, including toxicity avoidance, and overall survival [21, 26]. This debate makes multiple myeloma a “multiple approach” disease, as expresses itself through multiple possible treatment related guidelines. Briefly, the following classes of agents are available: novel agents, i.e. immunomodulatory drugs thalidomide, lenalidomide and pomalidomide and proteasome inhibitors bortezomib and -recently- carfilzomib, alkylating agents melphalan and cyclophosphamide, anthracyclines adriamycin and liposomal doxorubicin, and corticosteroids dexamethasone and prednisone. Other agents include platinum, vincristine, and etoposide, employed in aggressive combinations [4, 21, 27-28]. More molecules are expected to be introduced in treatment protocols, for example antibodies (anti-CD38

daratumumab and SAR650984, anti-CD319 elotuzumab, anti-IL6 siltuximab, anti-BAFF tabalumab, anti-RANKL denosumab, anti-sclerostin romosozumab) and heat shock protein inhibitors [26]. The most immediate classification of myeloma subjects is based on age and clinical conditions. Multiple myeloma cases are usually divided into two big classes: transplant eligible (<65 years or fit patients in good clinical condition) and transplant ineligible patients [4, 21, 26]. In the ESMO clinical practice guidelines [4], this distribution of myeloma patients is sufficient to elaborate a therapeutic strategy: in transplant ineligible patients oral combinations of melphalan and prednisone plus novel agents, in transplant eligible patients induction (3 drugs regimens based on bortezomib in combination or not with lenalidomide and thalidomide) followed by high-dose therapy (melphalan) before ASCT [4, 29]. In both types of approach, consolidation and maintenance therapy after induction or ASCT are not systematically indicated [4]. In addition to simple distinction of transplant eligible and ineligible patients, more sophisticated risk stratification systems based on host, tumor burden and tumor biology related factors have been elaborated. Patients with active disease may be categorized through the use of Durie-Salmon [30] and ISS [31] staging systems.

Table1 . ISS staging system from Greipp PR et al. International staging system for multiple myeloma. J Clin Oncol 23(15), 2005:3412-20. Erratum in: J Clin Oncol 23(25) 2005:6281. Harousseau, Jean-Luc [corrected to Avet-Loiseau, Herve]. [31]

Stage	Criteria	Median Survival (months)
I	Serum β_2-microglobulin <3.5 mg/L Serum albumin ≥3.5 g/dL	62
II	Neither stage l nor stage lll*	44
III	Serum β_2-microglobulin ≥5.5 mg/L	29

* There are two categories for stage II: serum β_2-microglobulin < 3.5 mg/L but serum albumin < 3.5 g/dL; or serum β_2-microglobulin 3.5 to < 5.5 mg/L irrespective of the serum albumin level.

As indicated in Durie and Salmon [30], classification of myeloma cases at diagnosis may be performed analyzing hemoglobin, calcium and M-protein levels. In Durie-Salmon staging system three stages are distinguished: I, characterized by hemoglobin >10 g/dL, serum calcium normal (≤12 mg/dL), normal bone structure or solitary bone plasmacytoma only, and low M-component production rate (IgG <5 g/dL, IgA <3 g/dL, urine light chain M-component on electrophoresis <4 g/24 h); III, associated with one or more parameters among hemoglobin <8.5 g/dL, serum calcium >12 mg/dL, advanced lytic bone lesions, and high M-component production rate (IgG >7 g/dL, IgA >5 g/dL, , urine light chain M-component on electrophoresis >12 g/24 h); II, fitting neither stage I nor stage III [30]. A subclassification was defined depending on renal function: A, normal renal

function (serum creatinine <2.0 mg/dL); B, abnormal renal function (serum creatinine level ≥2.0 mg/dL) [30]. In a subsequent study by Greipp et al. [31], median survival according to Durie-Salmon staging system was: 62 months for IA, 22 months for IB, 58 months for II A, 34 months for IIB, 45 months for IIIA and 24 months for III B [31]. ISS staging system and median survivals are reproduced in Table 1 [31]. In a further investigation, none of the two systems was recognized as strongly predictive of outcomes [32]. A complementary approach consist in the evaluation of cytogenetic, karyotypic and molecular abnormalities. Nearly half of MGUS and myeloma cases are hyperdiploid (HRD), characterized by 48–75 (mostly 49–56) chromosomes, usually with extra copies of three or more specific chromosomes (trisomies of 3, 5, 7, 9, 11, 15, 19 and 21 chromosomes). In turn, non-hyperdiploid (NHRD) tumors have <48 and/or >75 chromosomes. HRD tumours rarely (~10%) have a primary immunoglobulin heavy-chain (IgH) translocation; NHRD tumours usually (~70%) have an IgH translocation. HRD patients have a better prognosis than NHRD patients [33-35]. A total of 7 recurrent chromosomal partners and oncogenes involved in primary IgH translocations in MGUS and myeloma cases has been described. These alterations are divided into 3 translocation groups -chromosomal site, involved oncogenes, and prevalence in multiple myeloma are reported as described by Chesi and Bergsagel [33] and subsequent records [34-35]-: (**1**) CYCLIN Ds: 11q13 (*CCND1*) 15%; 12p13 (*CCND2*) < 1%; 6p21 (*CCND3*) 2%; (**2**) MAFs: 16q23 (*MAF*) 5%; 20q12 (*MAFB*) 2%; 8q24.3 (*MAFA*) < 1%; and (**3**) MMSET/FGFR3 4p16 (*WHSC1/MMSET*, and in 80% of cases also *FGFR3*) 15%. Secondary oncogenic events driving MGUS and multiple myeloma progression include: MYC dysregulation; chromosome 13 deletion; activating mutations of RAS, BRAF and NF-κB pathway; chromosome 17p loss and abnormalities of TP53; secondary Ig translocations; gain of chromosome 1q and loss of chromosome 1p [33-35]. The interest in patterns of expression of deregulated genes has led to analysis of gene-expression profiling (GEP) by different research groups. This was put into practice through the elaboration of various GEP models [36], the most known being GEP70 (developed by researchers at University of Arkansas)[37] and GEP15 (proposed by the Intergroupe Francophone du Myélome) [38], and GEP-based risk stratification systems [36-39]. Although GEP analysis is included in the algorithms for prognostic factors and risk stratification, the consensus guidelines do not openly recommend performing GEP analysis in a non-research or routine setting [27]. On these basis, the European Myeloma Network [26] presented a risk stratification system based on two categories, standard-risk and high-risk, and three types of determinants: host factors, tumor burden and tumor biology [26]. As reported by Engelhardt et al. [26], standard risk patients are characterized by Karnofsky Performance Status [40] (KPS) >70%, normal renal and organ function, no impairment in Geriatric Assessment (GA) [41], Freiburg Comorbidity Index (FCI) [42] and Charlson Comorbidity Index (CCI) [43] equal to 0 (all defined host factors), Durie-Salmon stages I and II (tumor burden), ISS stages I and II, hiperdiploidy, t(11;14), and t(6;14) (all tumor biology). In turn, high-risk patients present KPS <70%, estimated glomerular filtration rate

<30, organ impairment, reduced GA, advanced age (all host factors), Durie-Salmon stage III (tumor burden), ISS stage III, t(4;14), t(14;16), t(14;20), 17p-, 1q/del1p, GEP high-risk signature, high lactate dehydrogenase levels, and other aggressiveness determinants (presentation as plasma cell leukemia, high proliferation rates, presence of extramedullary disease) (all tumor biology). [26]. Of note, no specific association with eligible therapies was reported, but only indications about intensity of treatment and preferential use of bortezomib for patients with some tumor biology high-risk features, such as t(4;14) or del17p, were pinpointed [26]. Despite treatment suggestions provided with the scheme, therapeutic approaches remain based on the initial distinction of transplant ineligible and eligible patients: in transplant eligible, the strategy consists of induction with 3 drug regimen (including at least one among bortezomib, lenalidomide and thalidomide), followed by consolidation (considering second transplant in patients who did not reach at least very good partial response) with suggested maintenance therapy, especially bortezomib based therapy, in patients who failed to reach complete response or very good partial response after ASCT; in transplant ineligible patients, addition of a new drug to melphalan and prednisone containing regimens, with following maintenance therapy is recommended [26]. The IMWG combined ISS-genetic prognostic system proposed as a standard for risk stratification of myeloma patients recognizes 3 categories [28]. High-risk patients present ISS stages II/III, and t(4;14) or 17p13 deletion; low-risk patients are characterized by ISS stages I/II and absence of t(4;14), 17p13 deletion and gain of 1q21, and age <55 years; all other subjects are categorized as standard-risk [28]. No specific therapy is suggested by Chng et al. [28], with the exception of bortezomib based treatment for patients with t(4;14); the choice of therapeutic approach is left to physicians, taking into account the cited cure vs control debate [28]. A risk adapted therapeutic strategy was elaborated at the Mayo Clinic [21, 27, 44]. The update to risk stratification using mSMART classification (as indicated in Vincent Rajkumar 2014) is depicted in Table 2 [21]. Also high levels of lactate dehydrogenase and evidence of plasma cell leukemia on peripheral blood smear analysis are associated with high-risk disease [21]. The risk adapted therapeutic approaches are reported in Figure 1 [21, 44]. Patients with standard-risk disease exhibit a median survival of 6 to 7 years, whereas patients with high-risk and intermediate-risk disease show a median survival of 2 to 3 years and 4 to 5 years respectively [21, 27]. Another possible approach is allogenic stem cell transplantation, which is investigational and currently pursued in clinical trials [21, 26]. This paragraph has not the presumption to clarify all possible experts' positions about influence of prognostic factors in therapy choice. A detailed description of results inherent to ongoing clinical trials about molecular subgroup adapted therapeutic protocols were not included since no clear recommendation may be given [21, 26]. Moreover, such a type of in-depth report is not in line with the topic of this thesis. The objective of this brief dissertation is emphasizing the absence of a global consensus as regards the estimation of the impact of clinical and molecular data on definition of prognosis and administered drug protocols.

Table 2. Risk-stratification of myeloma as defined in mSMART classification

Standard-risk
Trisomies (hyperdiploidy)
t (11;14)
t (6;14)
Intermediate-risk
t (4;14)
High-risk[a]
17p deletion
t (14;16)
t (14;20)
High risk gene expression profiling signature

[a]In the presence of concurrent trisomies, patients with high risk cytogenetics should be considered standard-risk.

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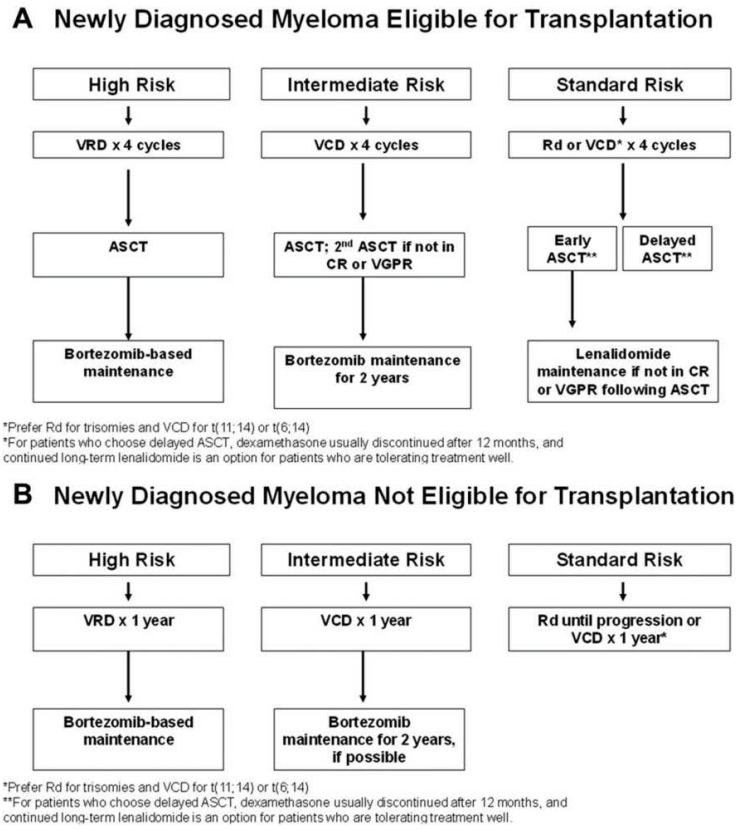

Figure 1. Risk adapted treatment of newly diagnosed myeloma in patients eligible for transplantation (A) and not eligible for transplantation (B). Rd, lenalidomide plus low-dose dexamethasone; Dex, dexamethasone; CR, complete response; VGPR, very good partial response; VCD, bortezomib, cyclophosphamide, dexamethasone; VRD, bortezomib, lenalidomide, dexamethasone; ASCT, autologous stem cell transplantation.

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1.2.3 Monitoring of response and impact on prognosis.

To assess response to therapy and disease progression, historically many categories has been proposed; nowadays, usually (but not universally) the used criteria are those elaborated by the International Myeloma Workshop Consesus Panel 1 [45], that adopted the International Myeloma Working Group (IMWG) uniform response criteria [46-47] adding clarifications, with some parameters inherited from the European Group for Blood and Marrow Transplantation (EMBT) [48]. These criteria were summarized by Bird et al. [25]. Complete response (CR) requires negative immunofixation of serum and urine, disapparence of any soft tissue plasmocytoma and <5% of plasma cells in bone marrow. When the only measurable disease is by serum FLC levels, a normal FLC ratio must be reported in addition to negative immunofixation [25, 45, 47]. The International Myeloma Workshop Consesus Panel 1 [45] introduced the definition of molecular CR is indicated as CR plus negative ASO-PCR (sensitivity 10^{-5}) [45]. Stringent complete response (sCR) was updated by the International Myeloma Workshop Consesus Panel 1 [45] as CR plus normal serum FLC ratio, and absence of clonal plasma cells by immunohistochemistry or MFC (originally immunohistochemistry or immunofluorescence) [25, 45-47]. Immunophenotypic CR, as approved by International Myeloma Workshop Consesus Panel 1 [45], requires criteria of sCR plus absence of clonal plasma cells in bone marrow with a minimum of 1 million total bone marrow cells analyzed by MFC (with ≥4 colors) [45].Very good partial response (VGPR) implies that serum M-component is detectable only by immunofixation (not on electrophoresis), or ≥90% reduction in serum M-protein with reduction in 24-h urinary M-protein to <100 mg/24 h. When SFLC assay is used to monitor disease response, VGPR is defined by >90% decrease in the difference between the involved and uninvolved FLC concentration [25, 45, 47]. Partial response (PR) includes ≥50% reduction of serum M-protein and reduction in 24-hour urinary M-protein by ≥90% or to <200 mg/24 hours; if the serum and urine M-protein levels are not measurable, a decrease ≥50% in the difference between involved and uninvolved FLC levels is necessary. If FLC levels are unmeasurable too, ≥50% reduction in bone marrow plasma cells is required (provided baseline percentage was ≥30%). In addition, if present at baseline, ≥50% reduction in the size of soft tissue plasmacytomas is also indispensable to confirm a PR [25, 45-47]. Stable disease (SD) is characterized by absence of meeting criteria for CR, VGPR, PR or progression of disease [25, 45-46]. Progressive disease (PD) is the most articulated category. It requires any of the following: ≥25% increase in serum M-protein (absolute increase ≥5 g/l); ≥25% increase in urine M-protein (absolute increase must be ≥200 mg/24 h); ≥25% increase in the difference between involved and uninvolved FLC levels (only in patients without measurable serum and urine M-protein levels; absolute increase >100 mg/l); ≥25% increase in bone marrow plasma cell percentage (only in patients without measurable serum and urine M-protein levels and without measurable disease by FLC levels; absolute percentage of bone marrow plasma cells ≥10%); development of new bone lesions or soft tissue plasmacytomas or increase in the size of existing bone lesions or soft tissue

plasmacytomas; development of hypercalcemia [25, 45]. In turn, minimal response (MR) (for patients with relapsed and/or refractory myeloma) consists of ≥25% but ≤49% reduction of serum M protein and reduction in 24-hour urine M-protein by 50%-89%, 25%-49% reduction in the size of soft tissue plasmacytomas if present at baseline, and no increase in size or number of lytic bone lesions, as re-elaborated recently [45, 48].

On these basis, the International Myeloma Workshop Consesus Panel 1 [45] described relapsed and refractory myeloma (nonresponsive while on salvage therapy, or progresses within 60 days of last therapy in patients who have achieved MR or better before progressing), primary refractory myeloma (nonresponsive in patients who have never achieved a minimal response or better with any therapy) and relapsed myeloma (previously treated myeloma that progresses and requires the initiation of salvage therapy without meeting criteria for "primary refractory myeloma" or "relapsed-and-refractory myeloma") [45].

An increasing number of studies have been performed in both transplant and non-transplant settings to detect the prognostic role of the depth of response on prognosis. Resuming all these data and organizing them in a comprehensive form in this thesis would be misleading, and would open a complex debate on inter-study difference in significant results. A panel of factors may compromise efficient comparisons of the studies: different definition of CR, dishomogeneity of evaluated treatments, analysis of small subsets of patients, type of comparison – in some studies patients with CR or CR plus VGPR are compared with partial remission patients, in others they are compared with more heterogeneous groups of subjects, including patients with SD or PD [49]. Leaving behind all possible reasons which make the comparisons of existing results difficult, a critical review of literature permits to conclude that improvements in the quality of response (as depth and durability) are generally associated with longer survival [28, 49-50]. Thanks to new therapeutic strategies, both transplant eligible and ineligible patients are now able to reach quality responses (markedly improving CR rates), in all phases of treatment [50]. The association of CR and VGPR with improved survival is better defined in transplant setting, but lesser explored in transplant ineligible patients [51-55]. Despite this premise, definitive data to validate the association between quality of response and survival outcomes are missing. The ability to sustain a deep response is becoming a crucial point to debate, since conflicting reports indicate that some patients may be able to achieve deep but short responses, while others reach durable MR [28, 50]. Anyway, the objective of achieving a maximal response should always be balanced with tolerability and patient perceived quality of life [49-50]. All these considerations recall the "cure versus control" debate treated in the paragraph 1.2.2. Clearly, the situation may be improved by intensive use of technologies which allow predictive evaluation about the type of response a patient will be able to reach, and estimation of the probability of relapse [50-51]. In this sense, progressions were recorded for MFC approaches [49-50, 56], as I discuss in the following sections. However, a universally recognized solution is still far.

1.3 MFC applications in multiple myeloma and MGUS: when, how and why.

MFC utility extends from diagnosis to MRD assessment, monitoring of patients and definition of response [15, 45, 57-58]. Prognostic role of MFC immunophenotyping is still waiting for a universally validated correlation with therapeutic decisions [15, 59-60]. In addition, MFC may be used to assess plasma cell labeling index (PCLI, proportion of plasma cells in S-phase) and ploidy through calculation of DNA index (DNA content of neoplastic cells/DNA content of normal cells in the sample), to deepen the study of documented bone marrow population-microenvironment alterations related to disease esordium and progression, and to explore the modifications in peripheral blood leukocytes [3, 15, 61-67]. Other possible uses of MFC (which are unrelated to this thesis and for which I cross-refer to specific resources) include definition of new therapeutic targets [68], characterization of circulating plasma cells [69], study of the effect of therapeutic regimens on antigenic expression [70-71], identification of clonogenic compartment [72-75], quality graft assessment and enumeration of CD34+ HPCs in peripheral blood after mobilization [76].

1.3.1 General considerations on MFC plasma cell detection.

MFC analysis of big amounts of cells in the same sample (in a short period of time), added to the possibility of simultaneous analysis of surface and intracellular antigens, allows identification, characterization and enumeration of even small percentages of plasma cells in both multiple myeloma and MGUS samples [15, 77]. Lower bone marrow plasma cell counts have been found during MFC vs morphological analysis through conventional microscopy; these discrepancies affect also cytogenetics/FISH and molecular investigations [15, 57, 77]. The most relevant reasons for this inequality are the highest quality of “first-pull” bone marrow aspirates used for morphological evaluation (whereas secondary specimens are generally used for other laboratory purposes) and technical issues related to morphological enumeration of plasma cells (begin of enumeration from fields containing higher numbers of plasma cells rather than on randomly chosen fields and/or focus on bone marrow particles with an increased concentration of plasma cells) [15, 57, 77]. Also plasma cell fragility may play an important role in causing methodological diversity of results [57]. However MFC quantification of bone marrow plasma cells is strongly suggested in clinical routine. It has been demonstrated that plasma cell counts obtained with MFC and morphological examination exhibit a significant positive correlation [78]. Paiva et al. [78] demonstrated that patients with bone marrow plasma cells < 15% as detected by MFC showed also prolonged overall survival than cases with bone marrow plasma cells ≥ 15% (median 97 vs 54 months, respectively); at multivariate analysis, bone marrow plasma cell counts obtained by multiparameter flow cytometry were confirmed as an independent prognostic factor for overall survival [78].

MFC identification of plasma cells relies on the combined use of CD38, CD138, CD45 and light scatter characteristics, setting the first gate in the bivariate dot plot CD38vsCD138 as reported by the European Myeloma Network [77].

No single surface marker can make the distinction between normal and clonal plasma cells [58]. A repertoire of studies indicated that the immunophenotype of clonal/aberrant plasma cells differs from that of normal/reactive polyclonal plasma cells as regards a discrete number of markers, which are routinely employed for characterization of myeloma cells and are also used in this thesis (i.e. CD45, CD19, CD56, CD117, CD27, CD20, CD200) [18, 57-58, 77, 79-80]. A minimum MFC panel should contain antibodies to test the surface expression of (at least) CD138, CD38, CD56, CD19 and CD45 [77, 79]. Assessment of clonal restriction through cytoplasmic staining of light chains is usually used to confirm adequate discrimination of aberrant plasma cells from polyclonal counterpart [15, 18, 57-58, 77]. At diagnosis, the presence of >5% residual polyclonal plasma cells (of total bone marrow plasma cells) is accepted as a reliable parameter to distinguish MGUS from multiple myeloma cases [15, 81-82]. In the following sections the dissertation is deepened focusing on classical characterization markers which have been studied in this thesis.

1.3.2 Challenges in plasma cell detection and myeloma cell discrimination.

Phenotypically aberrant plasma cells are typically CD19 negative (~95% of cases), CD45 negative (~70% of patients), CD27 weak or negative (~40-50% of cases), CD56 positive (~60-70% of subjects) and CD200 weakly/strongly positive (~70% of patients); in selected cases, CD20 is heterogeneously expressed -negative to positive- (~17%) or positive (~30%) and CD117 is reported positive (~30%) [15, 57-60, 77, 80, 83-86]. Comparing different studies, a negligible degree of variability in frequency of these immunophenotypic characteristics is observed [15, 57-60, 77, 80, 83-86]. On the contrary, normal plasma cells were usually described as CD19+ (>70% of cells), CD27+ (~100% of cells), CD45+ (with fractions of CD45- plasma cells, ~6%), CD200 weakly positive or negative, CD20- (with fractions of CD20dim plasma cells, ~4%) and CD56- (CD56+ plasma cells <15%) [15, 58-59, 77, 80, 87]. Although some groups suggested the use of simplified antibody panels to distinguish neoplastic plasma cells from normal/reactive plasma cells [79], recent reports demonstrate that features of “normal” and “aberrant” phenotypes are becoming less solid with improvements in gating and detection strategies, and the abandon of cut-off values to define positivity. In fact, normal plasma cells have been demonstrated to be more immunophenotypically heterogeneous than previously understood, and subpopulations of non-neoplastic plasma cells with an immunophenotype similar to myeloma plasma cells (CD20+CD56+CD45-CD19-) have been described [85, 88-91]. As a consequence, in these last years the scenario has been changing dramatically as regards ranges of expression of classical immunophenotypic markers on normal plasma cells; the most cited papers and related disclosures are indicated in Table 3 [57-58, 77, 87-93]. Precedent studies explored immunophenotype of

polyclonal plasma cells only in healthy individuals [87, 90], while others reunited in the same category normal plasma cells of healthy and pathological subjects [88, 94], causing a loss of information related to possible normal plasma cell heterogeneity among different categories of patients.

It is not fully demonstrated if normal plasma cells in post therapy bone marrow may exhibit immunophenotypic variations interfering with MRD detection, as well as if such (eventual) variations may have a utility in diagnosis/monitoring of disease or for prognostic purposes. Post therapy immunophenotypic changes have been demonstrated in clonal plasma cells [95-96], but it is not clear if disease progression or response to therapy may be predicted analyzing plasma cell ability to downregulate or upregulate surface antigen expression during treatment.

Table 3. Ranges of expression for classical immunophenotypic markers as reported on normal plasma cells

	Range for positive cells (%)	Range for negative cells (%)
CD45	41-100 (Ref. 89)	
CD19	61 - 100 (Ref. 87) 44.2 - 77.1 (Ref. 90)	7 - 50 (Ref. 88)
CD56	0 - 47 (Ref. 87) 5.7 - 41.8 (Ref. 90) 5 - 22 (Ref. 88) <15 (Ref. 77) 10 - 100 (Ref. 91) 0 - 37 (Ref. 89)	
CD117	0 (Ref. 57-58, 77, 87)	
CD27	66 - 100 (Ref. 87) >98 (Ref. 92) 100 (Ref. 77)	
CD200	0 (Ref. 59) 0 (Ref. 87) W Pos (not specified) (Ref. 77) Pos (Ref. 93*)	
CD20	19 - 91 (Ref. 87) 13 - 37 (Ref. 88) 0 (Ref. 77)	

*Study not conducted on bone marrow. W Pos, weakly positive; Pos, positive – no specified range.

The current list of MFC markers for myeloma and MGUS plasma cell evaluation remains limited if compared to other clonal hematopoietic disorders and it has not augmented significantly in the past years [15-16, 18, 57]. Moreover, not all surface antigens exhibit stable expression in samples; it is the case of CD138, whose time- and apoptosis-dependent loss from plasma cell surface has been previously demonstrated [97-98]. In addition, the introduction of new therapeutic approaches targeting classical MFC surface markers with monoclonal antibodies (i.e. against CD38 or CD138) [99-101], may compromise the utility of these molecules for after-treatment follow-up, limiting

their value in plasmacellular detection, and the resolution power of current combinations; similar experimental conclusions were described in other hematological malignancies and/or considering other types of monoclonal antibodies [101-102].

Thus introduction of new markers in clinical routine is highly recommendable. These markers should be suitable for discrimination of clonal plasma cells from their normal counterpart, and for identification of plasma cells in absence of CD38 and CD138.

1.3.3 MFC in assessing prognosis

In recent years, an increasing interest about the role of MFC in establishment of prognosis has started to appear; however, frequent discrepancies have been reported among various reports [15, 57, 60, 80]. This is probably due to an inappropriateness in study design and technical pitfalls, just for example the use of different clones of monoclonal antibody conjugates, and adoption of different cut-off values for definition of positivity [60]. Thus the prognostic value of immunophenotyping in MGUS and multiple myeloma, although being accepted, needs validation.

Besides the utility of MFC enumeration of plasma cells at diagnosis (paragraph 1.3.1), a correlation with prognosis has been made for an extended number of MFC markers [15, 57-58, 80]. As reported by Mateo et al. [60] CD19 positivity and CD117 negativity associated with shorter progression free survival compared with CD19- and CD117+ patients (median 26 vs 38 months and 32 vs 44 months respectively). Similar considerations emerged as regards overall survival. For CD19+ cases median overall survival was described 40 vs 68 months of CD19- patients [60]. Bataille et al. [103] affirmed that overall survival was 93% at 4 years in CD117+ vs 64% in CD117− myeloma patients [103]. Lack of CD117 was associated with high levels of β2-microglobulin, advanced ISS, non-hyperdiploid cases (or more specifically hypodiploidy), t(11;14) and t(11;4) traslocations and del(13q) [60, 104]. CD20 expression has been associated with t(11;14) [84, 86], although CD20 expression did not show any impact on survival [60]. Data about CD45 are incoherent. It has been reported that CD45 has no impact on prognosis [60], despite the association of CD45- cases with deletion 13 or aneuploidy and del(17p) by FISH [104], and the trend towards longer overall survival for CD45+ (defined as >20% of CD45 expressing plasma cells) patients vs the CD45- group (39 vs 18 months respectively), which did not reveal to be significant [105]. On the contrary, this trend related to the effect CD45 positivity on survival reached statistical significance in patients treated with high-dose therapy (median survival in CD45- patients 42 months vs median not reached in CD45+ patients) [106]. As regards CD27, Moreau et al. reported that overall survival was 92% at 3 years in CD27+ vs 50% in CD27– myeloma patients [107]. Moreover loss of CD27 is correlated to loss of CD19 in myeloma cases [92]. Sahara et al. [108] proved that median survival was lower in CD56- than in CD56+ patients treated with conventional chemotherapies (22 vs 63 months) and that CD56- cases are characterized by increased aggressiveness of disease; however authors failed to find any prognostic

value of CD56 expression at multivariate analysis, probably because of a strong association between CD56 expression and extramedullary disease [108]. In addition, CD56 showed no prognostic value in patients undergone to high-dose chemotherapy [109]. A correlation between CD56 negativity/down regulation on myeloma cells and t(11;14) has been described more than once in literature [84, 109]. Considering existing data, the role of CD56 in determination of prognosis needs clarification. Loss of CD200 expression in multiple myeloma was described as associated with the possibility of a more clinically aggressive disease [59]; this contradicts a precedent report based on Affymetrix microarrays, suggesting that patients with myeloma cells devoid of CD200 expression have a better survival than patients showing CD200 expression after high-dose therapy and stem cell transplantation [110]. So, also for CD200 data need to be deepen and better interpreted.

Other than on immunophenotypic characteristics, elaboration of prognosis relies on MFC quantitative data. Paiva et al. [111] demonstrated that, among myeloma patients, subjects with more than 5% normal plasma cells from total bone marrow plasma cells had clinical characteristics generally associated with a favorable outcome. These patients showed better response rates to both induction (rate of complete remission 21% vs 11% in patients with ≤5% of normal plasma cells out of total bone marrow plasma cells) and ASCT (rate of complete remission 64% vs 33% in patients with ≤5% of normal plasma cells out of total bone marrow plasma cells) [111]. Moreover, cases with more than 5% normal plasma cells from total bone marrow plasma cells exhibited a better outcome than patients with ≤ 5% normal plasma cells from total bone marrow plasma cells as regards both progression free survival (median 54 vs 42 months respectively) and overall survival (median not reached vs 89 months respectively) [111]. Examining a cohort of patients treated with high-dose therapy and ASCT, and assessing MRD by MFC after transplantation, progression-free survival and overall survival were longer in patients who were MRD negative versus MRD positive (progression free survival median 71 vs 37 months respectively, and overall survival median not reached vs 89 months respectively) [112]. Similarly Rawstron et al. [113] described that in patients undergone to ASCT, comparing MFC MRD negative with MFC MRD positive cases, a significant inferior outcome was reported for MRD positive patients vs MRD negative ones as regards progression free survival (15.5 vs 28.6 months) and overall survival (59 vs 80.6 months) [113]. Coherently, Paiva et al. [114] reported that in a cohort of CR patients, the failure to reach immunophenotypic CR at day +100 after high-dose therapy/ASCT produced a significant worse outcome concerning time to progression (at 3 years 58% for no immunophenotypic CR vs 86% for immunophenotypic CR) and overall survival (at 3 years 80% for no immunophenotypic CR vs 98% for immunophenotypic CR). Moreover at multivariate analysis, immunophenotypic CR status was confirmed as an independent factor able to predict unsustained CR (patients who lose their CR status within 1 year of high-dose therapy/ASCT) [114]. Considering patients who achieved CR with salvage therapy -with or without stem cell transplantation- after relapse, MFC MRD negative

patients (not undergone to allogenic stem cell transplantation) showed a median time to progression of 75 months vs 14 months in MRD positive patients [115]. MFC may also be helpful in assessing risk of progression towards symptomatic disease both in MGUS and SMM, as specified in paragraph 1.2.2. Analyzing MGUS subjects, the cumulative probability of progression was 25% for patients with ≥95% of clonal plasma cells (out of total bone marrow plasma cells) vs 5% for patients with less than 95% of clonal plasma cells (out of total bone marrow plasma cells) [116]. Also, in SMM cases, cumulative probability of progression at 5 years was 64% for patients with more than 95% of clonal plasma cells (out of total bone marrow plasma cells) vs 8% for patients with less than 95% of clonal plasma cells (out of total bone marrow plasma cells) [116]. Prognostic value of these results was confirmed in a further study [117]. In addition, CD19 showed a predictive value in MGUS, since Olteanu et al. [82] showed that MGUS patients with potential for disease progression seemed to lack CD19 expression on >90% of their plasma cells [82]. This overview emphasizes the deep connection between immunophenotypic characteristics of aberrant plasma cells and possible patient outcomes, also underlining the lack of homogeneity among results and the absence of information concerning normal plasma cells. In addition, the existing data indicate that depth of response to therapeutic intervention may exhibit a relationship with MFC results. Such a type of connection should be carefully considered in studies assessing the value of immunophenotypic profiles in order to complete the already delineated frame.

1.3.4 Exploring the interaction with bone marrow microenvironment part I - Immunosuppression

The complex interaction between myeloma cells and bone marow microenvironment, and the involvement of such interaction in myeloma pathophysiology, are the core of an expanding and stimulating field of research. The key question is how mechanism supporting hematopoiesis are manipulated by tumour cells in order to maintain their own growth. This process include both direct contact with bone marrow components and action of soluble mediators, with triggering of anti-apoptotic and pro-proliferative patterns, and appearance of immune dysfunction [3, 118]. Most of all, suppression of full immune functions is a recognized cause of increased risk of infection in multiple myeloma subjects [119-120]. Such a deterioration is related to not only intrinsic characteristics of the disease, but also to the effect of age dependent complications and the use of therapeutic approaches, which showed a documented impact at various levels on the immune system [119-120]. Myeloma-related immunodeficiency involves deficit in fuction of dendritic cells, and abnormalities in T cells (increase of CD4+ and CD8+ Tregs in myeloma patients vs normal subjects; CD4+ lymphopenia; inversion of CD4:CD8 ratio; abnormal Th1/Th2 CD4+ ratio; imbalance of T lymphocyte subsets), myeloid derived suppressor cells (MDSC) (including increase in granulocytic MDSC in patient with progressive disease), and NK cells [3, 63, 65-67, 118]. However, broad studies on deficits involving bone marrow B cell branch in myeloma subjects have not been performed yet. Most of data are about bone marrow alteration of total B cell percentage

and B progenitor cells [121-123], but distribution of B transitional/naïve and memory B cells in bone marrow among different categories of responding/relapsing patients still represent a critical point to deepen. B cell compartment is defective in the elderly: humoral immune response differs both in the quality (especially in terms of production of high affinity responses) and levels of the produced antibodies, and the number of circulating B cells significantly decreases in the aged [124-127]. In the elderly, naïve/transitional B cells are highly activated to produce both IL10 and TNF-α under physiological (anti-CD40 and IL4) stimulation; in turn, "double negative" (DN) IgD−CD27− population seems to be an "exhausted" memory population filling immunological space in aged subjects [127-130]. Bulati et al. hypothesized that DN B cells are involved in the inflammatory environment related to aging and that they might be either a by-product of systemic inflammation or directly involved in the immune response [129-130]. DN B cells show the ability of being stimulated to secrete granzyme B, and exhibit a tissue trafficking phenotype [129]. The reduction in percentage of naïve B lymphocytes (IgD+CD27−) and the increase in percentage of a DN memory B cell population have been demonstrated in peripheral blood of the elderly [128]. No experimental evidency about redistribution of these B cell subsets has been produced in the bone marrow of myeloma and MGUS patients.

1.3.5 Exploring the interaction with bone marrow microenvironment part II – Connection with the immunophenotype.

Residual clonal plasma cells may escape therapeutic effects in bone marrow niches, which has been proved to be able to enhance myeloma cell survival and modulate immune system ability to eradicate malignant cells [3, 33]. It has now become clear that, in order to cure multiple myeloma, targeting bone marrow players other than myeloma cells and identifying the role of bone marrow microenvironment in response to therapeutic intervention are necessary [3]. MFC allows determination of immunophenotypic profiles of neoplastic plasma cells, and simultaneous study of characteristics of other cellular populations in the same sample. This type of analysis may provide useful pieces of information about the possible connection between clonal plasma cell immunophenotype and bone marrow characteristics which may be related to outcome. Besides the immune system deregulation, another (influencing survival) field which is currently receiving growing attention is the analysis of graft content [76]. The CD34+ compartment is really heterogeneous, containing several subpopulations of hematopoietic progenitor cells (HPCs). In patients with myeloma and other hematological malignancies, studies about correlation between graft composition and durability of engrafment and neutrophil or platelet recovery time were performed especially as regards CD34+CD110+, CD34+CD133+, CD34+CD90+, CD34+CD38- and CD34+L-selectin+ cells [131-133]. Moreover, graft composition was explored comparing good and poor mobilizers, but no difference was detected about proportion of CD34+CD38− and CD34+HLA-DR− cells [134]. A report by Arber et al. [135] involving non-myeloma subjects

demonstrated graft-source dependent and donor-age dependent differences in progenitor subsets [135]. Given these data, the analysis of bone marrow CD34+ HPC subset distribution in myeloma patients would provide new insights in both relationship interesting medullary reserves-mobilized fractions and mechanism of reciprocal influences between clonal plasma cells and bone marrow niches. Precedent studies demonstrated alterations in bone marrow distribution of HPCs in multiple myeloma subjects. Compared to healthy donors, a substantial reduction of CD34+ HPC subsets and CD19+CD38+CD34+ Pro-B cells in terms of absolute cell count and proportion of mononuclear cells respectively was described in untreated myeloma subjects [122]. Similarly, percentage of total CD34+ cells and CD19+CD34+ cells (both defined as proportion of total leukocytes excluding plasma cells) was proven to be decreased at presentation, and CD19+CD34+ cells also at relapse, but not in patients at plateau/remission, vs normal individuals [121]. Coherently, a more recent report confirmed reduction of percentage of CD34+ HPCs (from whole bone marrow cellularity) and CD34+CD38+CD19+ progenitors (out of total CD34+ HPCs) in bone marrow of myeloma patients vs healthy controls [123]. However no specific analysis of myeloma patients was performed correlating immunophenotype of clonal plasma cells with bone marrow distribution of HPC subsets, with the exception of the work from Schmidt-Hieber et al. [136], who studied myeloma cases accordingly to clonal plasma cell CD117 positivity. The analysis demonstrated a significant higher CD34+CD19-/CD34+CD19+ cell ratio in bone marrow of newly diagnosed CD117- subjects vs CD117+ patients [136]. However, treated patients were not examined, and no intragroup comparison in CD117- and CD117+ responding and relapsing groups of patients was performed about distribution of CD34+ HPCs. Performing this analytical approach could reveal important features strictly dependent on mechanisms by which CD117 positivity is associated with a good prognosis, or open the research for a possible relationship of enumeration of bone marrow CD34+CD19- and CD34+CD19+ HPC subtypes with depth of response, mobilizing capacities, graft content and time to engrafment in CD117+ and CD117- patients. Moreover, it would make possible the evaluation of the effect of therapeutic regimens on CD34+ subpopulation reorganization in CD117- and CD117+ subjects.

2. Finding solutions: objectives of this thesis .

As emerged in previous paragraphs, one of the most urgent needs in multiple myeloma field is the introduction in clinical routine of new markers for discrimination of normal and clonal plasma cells, with simultaneous exact definition of normal plasma cell immunophenotype. In order to avoid loss of information or misinterpretation of results, and to provide the basis for future prognostic purposes, a rigorous approach should be used, exploring characteristics of MFC profiles in both clonal and polyclonal plasma cells by comparison of different categories of untreated, responding and non-responding patients.

With this in mind, in chapter 3 I explore the utility of some "old school" markers (CD19, CD45, CD56, CD27, CD117, CD20, CD200) and CD49d, CD58, CD11a and CD30 in identification of clonal and polyclonal plasma cells in MGUS, newly diagnosed and treated subjects. I focus my attention on markers involved in cellular adhesion (CD49d, CD11a and CD58), because a better understanding of quantitative changes in expression of adhesion molecules during the stages of the disease may be fundamental for defining the mechanisms by which plasma cells moves to or from the BM niches [91]. I also analyze the expression of CD30, which is present normally on only a very small fraction of activated lymphocytes, contributes to negative selection of T-cells, and could be an attractive target for therapeutic intervention [137]. CD49d, the integrin α subunit of VLA-4, has been found strongly expressed by myeloma plasma cells with a median percentage of positive cells close to 100% [138], whereas mean percentage of positive normal plasma cells from healthy donors has been reported ~75% [70]. CD11a, the integrin α subunit of LFA-1, has been reported positive as well as negative in literature on MGUS and myeloma samples [139-140]. The ranges of expression on malignant plasma cells differs dramatically from one paper to another, ranging from 0 to 100% in newly diagnosed or remitting subjects, and from 0 to ~80% in relapsing subjects [91], or reported as <30% for CD45+ cells and <<10% for CD45- cells [105]. On normal plasma cells, it has been reported to range from 20 to 100% [91]. CD58, a CD2 receptor, was previously indicated as present occasionally on polyclonal plasma cells and never expressed on MGUS clonal plasma cells, but no clear range was defined through MFC analysis [139]. Similarly, CD30 expression on clonal plasma cells was recognized as infrequent, but no data are available as regards normal plasma cells [138]. For these markers, a prognostic role has not been defined yet. My primary endpoint is structured as follows: 1) analysis of the expression of CD45, CD19, CD27, CD56, CD117, CD20, CD200, CD49d, CD58, CD11a and CD30 in MGUS subjects, myeloma responding and relapsing patients, in order to test the utility of these markers in detecting clonal plasma cells in various clinical scenarios; 2) study of the differences in the immunophenotype of polyclonal plasma cells comparing normal subjects with responding and relapsing patients; 3) detection of variations in expression of the cited antigens on clonal plasma cells in groups of new or treated patients, and comparison with polyclonal plasma cells of MFC MRD negative and normal subjects [141]. As secondary endpoint I determine the existence of a correlation between exordium of disease/response to therapy/disease progression and the expression of the listed markers on

polyclonal and clonal plasma cells; then I verify if such a correlation is influenced by most commonly considered clinical and prognostic variables [141]. The inclusive objective is to define the utility of polyclonal plasma cell analysis in myeloma diagnosis and follow up, and the possibility of elaborating disease stage related MFC panels [141].

In turn, in chapter 4 I deepen the research of new immunophenotypic markers for detection of plasma cells. Critical revision of the literature resulted in a list of candidate markers to be evaluated: CD54, CD229, and CD319. CD229 and CD319 from SLAM family are self-ligand receptors [142], while immunoglobulin superfamily adhesion molecule CD54 has LFA-1 and Mac-1 as physiological ligands [143]. Their expression profile on malignant plasma cells has been previously described [68, 70, 144]; all of them have also been investigated as therapeutic targets for multiple myeloma patients [68, 144-145]. Despite their presence on myeloma plasma cell surface was documented in different experimental settings, none of the cited markers has been evaluated as alternative candidates to antigens currently used in MFC detection and characterization of plasma cells.

In chapter 5 I explore the interaction of the disease with bone marrow microenvironment, focusing on one of the most essential clinical aspects of multiple myeloma: immunosuppression. Since data about B cell branch are lacking, I concentrate my research on reorganization of B lymphocytes in myeloma patients. I report the distribution of B cell subsets in bone marrow of healthy subjects, MGUS, and newly diagnosed, responding and relapsing myeloma patients, also evaluating the possible presence of clonal restriction in these lymphocytic subpopulations through MFC.

Finally in chapter 6 I focus my attention on possible alterations interesting CD34+ HPCs in CD117+ and CD117- myeloma and MGUS patients. I verify the distribution of bone marrow CD34+CD19- HPCs and Pro-B cells in healthy controls, MGUS patients and various categories of responding/relapsing MM subjects. Moreover, after dividing patients accordingly to CD117 expression or absence on clonal plasma cell surface, I compare differences in percentage of CD34+CD19- and Pro-B cells to detect a potential mechanism related to influence of CD117 positivity and negativity on prognosis.

All material in this thesis is an original research; content of chapters 3 has been accepted on Cytometry Part B: Clinical Cytometry [141]. Content of chapter 5 and 6 has been submitted to a peer reviewed journal, while results in chapter 4 are part of manuscripts in preparation. The experimental activity described in chapters 3, 5 and 7 was performed at the U.O.S.D. Laboratorio Specialistico Oncologia, Ematologia e Colture Cellulari per Uso Clinico, ARNAS Civico (Palermo, Italy) from April 2013 to December 2013, from July to August 2014 and in December 2014. In turn, investigation exposed in chapter 4 was performed at the Centro de Investigación del Cáncer (Instituto de Biología Molecular y Celular del Cáncer, CSIC-USAL), Instituto Biosanitario de Salamanca (IBSAL), Servicio General de Citometría y Departamento de Medicina (NUCLEUS),

Universidad de Salamanca (Salamanca, Spain), from January 2014 to June 2014 and from September 2014 to November 2014.

3. Old and new immunophenotypic markers in multiple myeloma for discrimination of responding and relapsing patients: The importance of “normal” residual plasma cell analysis

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Original Article

Old and New Immunophenotypic Markers in Multiple Myeloma for Discrimination of Responding and Relapsing Patients: The Importance of "Normal" Residual Plasma Cell Analysis

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Background: Multiple myeloma is an incurable disease characterized by proliferation of clonal malignant plasma cells (CPCs), which can be immunophenotypically distinguished from polyclonal plasma cells (PPCs) by multiparameter flow cytometry (MFC). The utility of PPCs analysis in detecting prognostic and predictive information is still a matter of debate. Methods: we tested the ability of 11 MFC markers in detecting differences in the immunophenotype of CPCs and PPCs among patients in various disease stages; we verified if these markers could be associated with disease stage/response to therapy despite the role of clinical parameters. Results: significant changes in the expression of markers occurred both in CPCs and PPCs. CD58 on PPCs of responding patients was downregulated compared with PPC of relapsing group. Fraction of CD200 expressing PCs was lower in control subjects than in PPCs from MGUS and myeloma groups. CD11a levels of expression on both CPCs and PPCs showed an upregulation in newly diagnosed and relapsing patients versus PCs of controls; CD20 was less expressed on control PCs than on MGUS CPCs and PPCs. CD49d revealed to be advantageous in discrimination of PPCs from CPCs. In our multiple regression model, CD19 and CD49d on CPCs, and CD45, CD58 and CD56 on PPCs maintained their association with groups of patients independently of other prognostic variables. Conclusions: we provide a feasible start point to put in order ranges of expression on PPCs in healthy and myeloma subjects; we propose a new approach based on PPC analysis to monitor the stages of the disease. © 2014 International Clinical Cytometry Society

Key terms: multiparameter flow cytometry; multiple myeloma; monoclonal gammopathies

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Additional Supporting Information may be found in the online version of this article.

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Multiple myeloma (MM) is an incurable neoplastic plasma cell (PC) disorder characterized by proliferation of clonal malignant plasma cells (CPCs) in bone marrow (BM), and presence of monoclonal protein (M protein) in blood and/or urine, associated with organ dysfunction (1). The first stage in the development of MM is the emergence of asymptomatic monoclonal gammopathy of undetermined significance (MGUS). In some of these patients, this progresses to smoldering MM and ultimately to symptomatic MM, with an annual risk of around 1% for patients with MGUS (2). MM accounts for approximately 1% diseases and is the second most common hematologic cancer. The incidence of MM in Europe is 4.5–6.0/100,000/year with a median age at diagnosis of between 63 and 70 years; the mortality is 4.1/100,000/year (3). Treatment differs among autologous stem cell transplantation eligible patients and those who are not candidates (4); responsiveness to treatments varies largely among patients due to the high heterogeneity of MM. The decision of which treatment is best has been a difficult issue in MM. However, due to the introduction of novel drugs (bortezomib, lenalidomide and thalidomide) that have been able to achieve good quality responses, changes in treatment strategies can be seen (5). The importance of reaching complete response (CR) is undoubtedly recognized in the transplant setting (6–8) although it is less clear in the nontrasplant setting. Clinicians may benefit from multiparameter flow cytometry (MFC), since this technology allows to define the immunophenotypic characteristics of CPCs, to study normal/reactive polyclonal PCs (PPCs) of both healthy and MM subjects, to distinguish CPCs from PPC pool (9–14), to evaluate the risk of progression from MGUS to MM (15,16) and the presence of minimal residual disease (MRD) (10,17,18), to identify new therapeutic targets (19–21), and to provide prognostic information. For some markers, immunophenotypic appropriateness and prognostic role have already been explored (CD27, CD56, CD117, CD19, CD45) (22–25), instead other markers have been less investigated (CD20 and CD200) (12,22,26,27) or their utility is still a matter of debate (CD30, CD49d, CD11a and CD58) (28–36). MFC data flank other factors influencing prognosis such as ISS and Durie-Salmon stage at diagnosis, hyper- and hypodiploidy and lactate dehydrogenase levels (37–39). A commonly described experimental approach is based on the study of immunophenotype of CPCs (and sometimes residual PPCs), dividing patients in newly diagnosed subjects and treated/relapsed patients. It is not fully demonstrated if PPCs in BM post therapies may exhibit immunophenotypic variations interfering with MRD detection; similarly it is not clear if disease progression or response to therapy may be predicted analyzing PC ability to downregulate or upregulate surface antigen expression during therapy. In addition, BM PPCs have been demonstrated to be more immunophenotypically heterogeneous than previously understood, and subpopulations of non-neoplastic PCs with an immunophenotype similar to myeloma PCs have been described (40–45). In this article, our primary endpoint was structured as follows: (1) analysis of the expression of CD45, CD19, CD27, CD56, CD117, CD20, CD200, CD49d, CD58, CD11a, and CD30 in MGUS subjects, MM responding and relapsing patients, to test the utility of these markers in detecting CPCs in various clinical scenarios; (2) study of the differences in the immunophenotype of PPCs comparing normal subjects with responding and relapsing patients; (3) detection of variations in expression of the cited antigens on CPCs in groups of new or treated patients, and comparison with PPCs of MRD negative and normal subjects. As secondary endpoint we determined the existence of a correlation between exordium of disease/response to therapy/disease progression and the expression of the listed markers on PPCs and CPCs; then we verified if such a correlation is influenced by most commonly considered clinical and prognostic variables. The inclusive objective is to define the utility of PPCs analysis in MM diagnosis and follow up, and the possibility of elaborating disease stage related MFC panels. In our knowledge, this is the first time that this kind of approach has been used to evaluate the expression of all these markers simultaneously in the same group of subjects.

MATERIALS AND METHODS

Patients and BM Samples

A total of 60 subjects (42 male and 18 female) were included in this study. Control specimens consisted of 10 BM samples from patients who were suspected to have a haematological disease and revealed to be non oncological subjects (Control group). These patients have no history of MM, MGUS or lymphoid/myeloid neoplasm. BM samples of 40 patients with MM and 10 patients with already documented (7 subjects) or newly diagnosed (3 subjects) MGUS submitted to our laboratory for routine analysis were evaluated by MFC and morphology. For every patient clinical chemical and immunological profiles, as well as reference intervals were provided by the U.O. Patologia Clinica – Laboratorio Analisi Cliniche of ARNAS Civico, Palermo (Italy). Clinical data and history for MGUS and MM cases were provided by U.O. Oncoematologia of ARNAS Civico, Palermo (Italy). Disease stage was defined according to Durie-Salmon and ISS staging criteria (46,47). Response to therapy was defined according to Bird et al. (48). Of MM samples, 10 were obtained at presentation (group New), 10 from patients with progressive disease (group Progressive), 10 from patients in partial remission (3 very good partial response, 6 partial response and 1 stable disease – group Therapy) and 10 from patients that achieved stringent CR (group Complete). All MGUS patients were considered as a separate group (MGUS group). Informed consent procedures and forms were proposed to and approved by the ARNAS Civico Medical Ethics Committee. Written informed consent was given by all subjects in line with the Declaration of Helsinki

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Table 1
Combinations of Antibodies

	FITC	PE	PC5.5	PC7	APC	APC-Alexa Fluor 750	PB	KO
Tube 1	Cyt κ	Cyt λ	CD38	CD56	CD138	CD27	CD19	CD45
Tube 2	CD20	CD56	CD38	CD117	CD138	CD27	CD19	CD45
Tube 3	CD11a	CD200[a]	CD38	CD56	CD138	CD27	CD19	CD45
Tube 4	CD30[a]	CD58	CD38	CD56	CD138	CD27	CD19	CD45
Tube 5	CD49d[a]		CD38	CD56	CD138	CD27	CD19	CD45

Cyt: cytoplasmic; FITC: fluorescein isothiocyanate; PE: phycoerythrin; PC5.5: phycoerythrin-cyanin 5.5; PC7: phycoerythrin-cyanin 7; APC: allophycocyanin; PB: pacific blue; KO: krome orange.
[a]All antibodies were purchased from Beckman Coulter (Miami, FL), except those with which were purchased from BD Pharmingen™ (San Jose, CA).

Protocol. BM samples were collected in EDTA tubes and processed in one hour since collection.

Multiparameter Flow Cytometry

Details about antibodies and instrument are indicated in on line Supporting Information Tables SI1 and SI2. Specimens were fragmented with a sterile syringe and filtered using a 80μm filter; nucleated cells were enumerated using UniCel® DxH™ 800 Coulter® Cellular Analysis System (Beckman Coulter, Miami, FL) and brought to a final concentration of 10^6cells/100μl with PBS w/o calcium and magnesium (EuroClone, Milan, Italy). Combinations of antibodies used to stain surface and intracellular markers are listed in Table 1. For staining of surface markers, 100μl of each sample were incubated with the opportune combinations of antibodies for 15 minutes in the dark. Erythrocytes were lysed adding 1 mL of VersaLyse™ Lysing Solution and incubating tubes for 20 minutes in the dark. For intracellular staining of kappa and lambda light chains, 50μl of sample were washed 5 times with 2 mL of PBS w/o calcium and magnesium (EuroClone, Milan, Italy), and processed with PerFix-nc (Beckman Coulter, Miami, FL) following instructions. Samples were all acquired with Navios™ Flow Cytometry System, data were collected with Navios v1.0 Software (Beckman Coulter, Miami, FL) and then analyzed with Kaluza® Flow Cytometry Analysis Software v1.2 (Beckman Coulter, Miami, FL). Daily testing of instrument was performed as indicated: standardization of light scatter, fluorescence intensity and optimal hydrodynamic focusing instrument settings were verified using Flow-Set Pro Fluorospheres (Beckman Coulter, Miami, FL); compensation matrix for each combination of antibodies was tested with CYTO-COMP™ Cell Kit (Beckman Coulter, Miami, FL); optical alignment and fluidics were checked using Flow-Check Pro Fluorospheres (Beckman Coulter, Miami, FL). To identify PCs, a combination of CD38, CD138, and CD45 together with side scatter properties was used; the first gate was set on CD38 versus CD138 as suggested (10). Distinction between normal/reactive and clonal PC compartments was performed on the basis of their most frequent aberrant phenotypes (as regards CD38, CD19, CD27, CD56, and CD45); results were confirmed by the presence of clonal restriction in population showing the abnormal phenotype, and the absence of restriction in normal PCs (10,22). The κ:λ ratio was defined as abnormal if < 0.5 or > 3 (49). A minimum of 200 events were collected in the PC gate for each tube; to reach this result, a total of 200,000–2,000,000 events were acquired. For each marker, an internal negative population present within the sample was used to define gates and sample fluorescence background (23,50). Data were measured as percentage of cells presenting the antigen (percentage of positive cells) and Mean Fluorescence Intensity Ratio (MFI ratio). MFI ratio for each fluorochrome-marker conjugate was defined as the geometric mean fluorescence of the positive population normalized for the geometric mean fluorescence of the negative population. Cellular DNA content and ploidy were analyzed by CYCLOSCOPE-MM (Cytognos, Salamanca, Spain) following instructions. DNA index was used to define the presence of aneuploidies as follows: hypodiploidy when the DNA index was <0.95, pseudodiploidy when the DNA index was 0.95–1.05 (excluding those subjects with DNA index of 1, who were indicated as perfect diploid), hyperdiploidy (HRD) when DNA index was >1.05, and near tetraploidy when DNA index was >1.75 (51).

BM Film Staining

BM films were prepared and stained with May-Grünwald-Giemsa staining method as described elsewhere (52,53). All reagents were supplied by Merck Millipore, Darmstadt, Germany.

Statistical Analysis

Continuous non normal data are expressed as median values (range); normal variables are indicated as mean±SD. Baseline differences between groups were assessed by the χ^2 test or Fisher's exact test, as needed for categorical variables. The univariate analysis of variance (ANOVA) was performed for parametric variables, and post hoc analysis with the Tukey's test was used to determine pairwise differences. The Kruskal-Wallis statistic test was performed for nonparametric analysis. The Wilcoxon signed-rank test was used to evaluate intragroup difference. For multiple comparisons the Bonferroni correction was performed. Multinomial logistic

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Table 2
General Characteristics of Subjects Included in This Study

		Control	MGUS	Complete	Therapy	New	Progressive		PW comp
Sex	M	100% (10)	70% (7)	40% (4)	80% (8)	30% (3)	100% (10)	Ct, P vs. N	0.02
	F		30% (3)	60% (6)	20% (2)	70% (7)			
Time fd (years)			7 ± 8.1 (7)	3 ± 1.8	1.5 ± 1.5	0 ± 0	4.4 ± 1.8	M vs. T	0.021
								M vs. N	0.002
Subtype	IgA λ		30% (3)			30% (3)			NT
	IgA κ				10% (1)		50% (5)		NT
	IgG λ		30% (3)	20% (2)	30% (3)	60% (6)			NT
	IgG κ		50% (5)	40% (4)	40% (4)	10% (1)	50% (5)		NT
	λ			40% (4)	20% (2)				NT
	κ			20% (2)					NT
D-S Stage	I A			20% (2)		30% (3)	70% (7)	T vs. P	0.003
	II A			20% (2)	50% (5)	50% (5)	30% (3)		
	II B				10% (1)	10% (1)			
	III A			60% (6)	10% (1)			Cp vs. N, P	0.01
	III B				30% (3)	10% (1)			
ISS Stage	I			10% (1)	30% (3)	40% (4)			
	II			90% (9)	40% (4)	30% (3)	80% (8)	Cp vs. N	0.02
	III				30% (3)	30% (3)	20% (2)		
ASCT				60% (6)	50% (5)			Cp vs. P	0.01
								T vs. P	0.032

Continous variables are indicated as mean ± SD; other results are expressed as percentage of cases. The number of subjects is indicated between brackets. *Y*, years; Time fd, time passed from diagnosis of the disease; λ, myeloma secerning only lambda chain; κ, myeloma secerning only kappa chain; D-S stage, Durie-Salmon stage; ASCT, autologous stem cell transplantation; PW comp, pairwise comparison; Ct, Control; M, MGUS; Cp, Complete; T, Therapy; N, New; P, Progressive; NT, not tested. Everytime different pairwise comparison against one group gave the same *P* value, groups that were compared with that group are reported separated by a comma.

regression analysis examined the correlation between various patients groups (dependent variables) and markers measurements (independent variable) in simple and multiple regression models. χ^2 and Likelihood Ratio χ^2 statistics were used in assessing goodness of fit in Regression model. Data were analyzed by the Epi Info software (version 6.0, Centers for Disease Control and Prevention, Atlanta, GA) and IBM SPSS Software 21.0 version (IBM Corp., Armonk, NY). All *P*-values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of Patients

Characteristics of patients and statistical significant differences as regards sex, stage, and therapies are summarized in Table 2. Examining age, MGUS patients (70.9 ± 5.6 years) were significantly older than Control and Complete subjects (58.3 ± 5.8 and 59.8 ± 8.5 years, respectively; vs. Control $P = 0.008$ and vs. Complete $P = 0.027$). Progressive patients (80.3 ± 4.2 years) also presented a more advanced age compared with Control, Complete, and Therapy (61.9 ± 9.5 years) ($P < 0.0005$ in all cases), and with New subjects (66.3 ± 10.8 years, $P = 0.002$). When MFC analysis was performed, 40% of Complete, 30% of Therapy and 30% of Progressive patients have interrupted treatment from at least 15 days. Lenalidomide was the treatment of choice in 40% of Complete and 20% of Progressive cases. Thalidomide as monotherapy was administered to 20% of Complete subjects. Bortezomib-dexamethasone therapy was taken by 20% of Therapy and 50% of Progressive patients (Progressive vs. Complete $P = 0.032$). In Therapy group, 20 and 30% of subjects underwent bortezomib-cyclophosphamide-dexamethasone and bortezomib-thalidomide-dexamethasone regimens, respectively. Statistical analysis was extended to therapeutic regimens administered to patients before the time of this study. Frequencies of each type of therapeutic regimen was recordered. Significant differences were found for vincristine-melphalan-cyclophosphamide-prednisone (VMCP) therapy, only given in 50% of progressive cases (Progressive vs. Complete and Therapy $P = 0.032$), and bortezomib-thalidomide-dexamethasone (VTD) regimen, used in 20% and 50% of Complete and Therapy patients, respectively, (Therapy vs. Progressive $P = 0.032$). Clinical variables recorded for each group are indicated in Table 3. Patients in each group were also subgrouped depending on presence of serum albumin ≥3.5 or <3.5 g/dL, serum creatinine levels < or ≥2 mg/dL and M protein production rates low, high or comprised between 5 and 7 g/dL (for IgG) and 3 and 5 g/dL (for IgA), just as reported for Durie-Salmon and ISS staging criteria, but in these cases no significant difference emerged (data not shown). In addition, data about presence of eventual comorbidities were recorded. No significant differences were observed

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Table 3
Clinical Characteristics of Patients

	Control	Mgus	Complete	Therapy	New	Progressive	PW comp	
CRP > 0.3			20% (2)	40% (4)	40% (4)	100% (10)	P vs.Ct, M P vs.Cp	<0.0005 0.0007
B2M < 3.5	100% (10)	80% (8)	100% (10)	40% (4)	40% (4)		P vs.Ct, Cp P vs.M	<0.0005 0.0007
B2M ≥ 5.5		20% (2)		40% (4)	30% (3)	50% (5)	P vs.Ct, Cp	0.032
3.5 ≤ B2M < 5.5				20% (2)	30% (3)	50% (5)	P vs.Ct, M, Cp	0.032
HB < 8.5					10% (1)			
HB > 10	100% (10)	100% (10)	100% (10)	70% (7)	80% (8)	50% (5)	P vs.Ct, M, Cp	0.032
8.5 ≤ HB ≤ 10				30% (3)	10% (1)	50% (5)	P vs.Ct, M, Cp	0.032
BUN > 25				10% (1)	20% (2)	50% (5)	P vs.Ct, M, Cp	0.032
LDH > 530				20% (2)	20% (2)	50% (5)	P vs.Ct, M, Cp	0.032
S IFE+		100% (10)		100% (10)	100% (10)	100% (10)	Ct, Cp vs.all	<0.0005
U IFE+		50% (5)		80% (8)	30% (3)	80% (8)	N vs.Ct, Cp; P vs.Ct, Cp	0.0007
S AB FLC ratio (range 0.31–1.56)		100% (10)		50% (5)	100% (10)	100% (10)	M vs.Ct, Cp; N vs.Ct, Cp; P vs.Ct, Cp	<0.0005
U AB FLC ratio (range 2.04–10.37)		80% (8)		10% (1)	40% (4)	50% (5)	M vs.Ct, Cp M vs.T	0.0007 0.005

Results are expressed as percentage of cases, the number of subjects is indicated between brackets. CRP > 0.3, C-reactive protein > 0.3 mg/dL; B2M < 3.5, β-2-microglobulin < 3.5 mg/L; B2M > 5.5, β-2-microglobulin > 5.5 mg/L; 3.5 ≤ B2M < 5.5, 3.5 mg/L ≤ β-2-microglobulin < 5.5 mg/L; HB < 8.5, hemoglobin < 8.5 g/dL; HB > 10, hemoglobin > 10 g/dL; 8.5 < HB < 10, 8.5 g/dL<hemoglobin<10 g/dL; BUN > 25, blood urea nitrogen > 25 mg/dL; LDH > 530, lactate dehydrogenase > 530 IU/L; S IFE +, serum immunofixation positive; U IFE+, urine immunofixation positive, S AB FLC ratio, serum abnormal free light chain ratio; U AB FLC ratio, urine abnormal free light chain ratio; PW comp, pairwise comparisons; Ct, Control; M, MGUS; Cp, Complete; T, Therapy; N, New; P, Progressive; all, M, T, N, P. Everytime different pairwise comparison against one group gave the same *P* value, groups that were compared with that group are reported separated by a comma. Different pairwise comparisons giving the same *P* value are reported separated by a semicolon.

for chronic renal insufficiency, hypothyroidism, obesity, thalassemia trait, HCV, and HBV related chronic hepatitis (data not shown). Diabetes interested 20% of Complete, 40% of MGUS and 70% of Progressive subjects (Progressive vs. Control, Therapy and New $P = 0.003$ in all cases). Cardiopathy was a complication in 20% of MGUS and 50% of Progressive patients (Progressive vs. all other groups except MGUS $P = 0.032$). Hypertension was observed in 40% of complete and new, 50% of therapy and 80% of progressive subjects (progressive vs. control and MGUS $P = 0.007$; therapy vs. control and MGUS $P = 0.032$).

PCs Analysis and Intragroup Comparisons between PPCs and CPC

As evidenced by morphology, at the time of diagnosis mean percentage ± SD of PC was 6.4 ± 2.4% for MGUS, 29 ± 7.4% for Complete, 33.5 ± 14.7% for Therapy, and 29 ± 11.7% for Progressive (MGUS vs. complete and progressive $P = 0.001$; MGUS vs. Therapy $P < 0.0005$). Instead, at the time of this study, values changed as follows: 1 ± 0% for Control, 6 ± 2.1% for MGUS, 2.1 ± 1.6% for Complete, 10.4 ± 10.5% for Therapy, 20 ± 7.8% for New, and 29 ± 22.2% for Progressive (Progressive vs.Control, MGUS and Complete, and New vs. Control $P < 0.0005$ in all cases; MGUS vs. New $P = 0.049$; Complete vs. New $P = 0.005$; Therapy vs. Progressive $P = 0.003$). Median percentages of PCs (range) detected by MFC were 1.9% (1.4–2%) for Control, 1.5% (0.4–2.4%) for MGUS, 0.5% (0.05–0.7%) for Complete, 0.9% (0.2–13.5%) for Therapy, 8.7% (1.6–18.8%) for New and 3.8% (1.4–35.2%) for Progressive (Complete vs. Control $P = 0.015$; Complete vs. New and Progressive $P < 0.0005$ in both cases; Therapy vs. New $P = 0.036$). Median values (range) of CPCs of total PCs were 79.5% (64–87.2%) for MGUS, 64.8% (2.8–99.8%) for Therapy, 98.2% (89.5–99.6%) for New and 98.9% (91.3–99.1%) for Progressive (MGUS vs. New $P = 0.008$, vs. Progressive $P = 0.023$). In Complete and Control 100% of PCs were polyclonal, while observed median percentage values

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(range) for PPCs in the other groups were: 20.5% (12.8–36%) for MGUS, 35.2% (0.2–97.2%) for Therapy, 1.8% (0.4–10.5%) for New and 1.1% (0.9–8.7%) for Progressive (Control and Complete vs. New and Progressive $P < 0.0005$ in all cases; vs. Therapy $P = 0.018$). In Control and Complete group, 100% of PCs were perfect diploid (Control and Complete vs. all groups $P < 0.0005$). Pseudodiploidy was detected in 20% of MGUS, 10% of Therapy and 10% of New subjects; instead, hypodiploidy interested 20% of New patients. HRD was identified in 80% of MGUS, 90% of Therapy, 70% of New, and 100% of Progressive subjects (Therapy and Progressive vs. Control and Complete $P < 0.0005$ in all cases; Control and Complete vs. MGUS $P = 0.0007$ and vs. New $P = 0.003$). Ranges of expression and median values for each studied marker on CPCs and PPCs as revealed by MFC are indicated in Table 4. Levels of expression for all studied markers expressed as MFI ratio are reported in Table 5. The analyzed markers showed different utility in distinguishing CPCs from PPCs in MGUS, new, therapy and progressive groups, except for CD30 and CD11a, which never showed differences comparing normal and neoplastic populations. Results for intragroup comparisons between CPCs and PPCs in all groups are indicated in Table 4 (ranges of positive PCs), and in Table 5 (MFI ratio).

Definition of Normal Immunophenotype

In order to verify if PPCs are immunophenotipically different from one group to another, we compared the expression of all markers on PPCs from all groups. Results are summarized in Figures 1A–1T. PPCs showed a relevant grade of immunophenotypic heterogeneity among groups.

Differences among Control and Complete PCs and CPCs

To define how CPCs differ from normal/reactive polyclonal PCs found in subjects who do not present signs of clonal expansion, we compared the expression of all markers on PCs in Complete and Control groups with CPCs in MGUS, therapy, new and progressive groups. Results are summarized in Figures 2A–2V.

Regression Analysis

Multinomial logistic regression analysis was used to evaluate the relation existing between the expression of MFC markers (independent variables—considered as both percentage of positive cells and intensity of expression measured by MFI ratio) and disease status defined as the appartenence of each patient to one precise group (dependent variables). Significant results are summarized in Table 6. As reference categories, we chose Control in analyzing PPCs, and MGUS in studying CPCs, since MGUS is considered a "preneoplastic" condition. To test the hypothesis that the significant markers remained informative also considering common prognostic variables we performed a multivariate regression analysis; among parameters showing statistical significant differences in this study, we selected age, sex, Durie-Salmon, and ISS stages at diagnosis, ASCT and HRD, since they were considered highly reliable in determining prognosis, and allowed us to obtain the best fitting models. Other variables did not exhibit explicative power in our model (data not shown). Results are indicated in Table 6.

DISCUSSION

Since the 90's, multiparameter immunophenotyping has been providing relevant information for the diagnosis, classification and monitoring of hematological malignancies. Immunophenotypic differences between normal and neoplastic cells are essential in detecting MRD in myeloma when there are fewer than 5% of PCs by morphologic examination and a very small percentage of PCs to detect clonality by histological examination. Moreover MFC immunophenotyping may be useful to define potential prognostic markers and new therapeutic targets. MFC is perceived as highly dependent on expertise and is regarded to have limited reproducibility in multicenter studies, mostly because of the lack of standardization in data interpretation, analysis, and presentation, and limited evaluation of "new" vs. "classical" markers (54). Since larger complexity of the multivariate data analyses of both major and minor cell populations produced new insights in what is commonly known as "normal" phenotype (41,42), and MM and MGUS present both a strong component of interaction between neoplastic compartment and surrounding cells, including residual PPCs, there is the urgent need to review phenotypic differences among subjects. In this study, we propose an accurate approach to investigate the role of various immunophenotypic markers in discriminating CPCs from PPCs. We clearly demonstrate that PPCs are not equal among patients, showing a great variability in terms of percentage of positive cells and levels of expression of many antigens. Immunophenotypic differences in CPCs between MGUS and MM subjects, as well as changes in the immunophenotype of MM CPCs after therapy have been described (14,18,24,45); here we observe that these changes may also interest PPCs. We focused our attention on some markers involved in cellular adhesion (CD49d, CD11a, and CD58), because a better understanding of quantitative changes in expression of adhesion molecules during the stages of the disease may be fundamental for defining the mechanisms by which the PCs adhere to or detach from the BM niches (32). We also analyzed the expression of CD30, which is present normally on only a very small fraction of activated lymphocytes. CD30 contributes to negative selection of T-cells, and could be an attractive target for therapeutic intervention (55). The classic immunophenotype of myeloma PCs has been described as CD38 bright positive (dimmer than normal PCs), CD138 positive, CD19 negative, CD45 dim to negative and CD56 positive with CD20 and CD117 positivity in selected cases (10,22,23,40). However recent reports demonstrate that PPCs could display a CD20+CD56+CD45-CD19- phenotype (40–42,56). Precedent studies

Table 4
Ranges of Expression for the Studied Markers in All Groups

		% of positive CPCs			% of positive PPCs			
		Median	Min	Max	Median	Min	Max	*P* value
CD45	Control				98.9	54.7	99	
	MGUS	89.2	33.8	97.8	99.1	80.3	100	0.005
	Complete				92.2	76.5	99.9	
	Therapy	26.7	11.1	100	97.6	89.1	100	0.011
	New	87.2	12.7	99.6	98	0	100	
	Progressive	89.9	3.7	98.2	100	78.8	100	0.005
CD19	Control				72.4	58.9	87.4	
	MGUS		0			100		0.002
	Complete				50	33.5	86.6	
	Therapy	0	0	95.3	96.1	13.6	100	0.01
	New	2.8	0	95.6	100	0	100	
	Progressive		0			100		0.002
CD56	Control				4.8	4.7	5.6	
	MGUS	23	4.8	92.9	27.5	24.1	35.7	
	Complete				12	0	18.6	
	Therapy	95.1	0	100	10	0	100	0.05
	New	95.8	4.9	100	10	10	35.3	0.023
	Progressive	96.2	86.7	98.5	25.3	10	26.4	0.005
CD49d	Control				88.4	48.5	100	
	MGUS	48.7	42.9	88.6	75	74.2	91.9	0.012
	Complete				90.8	78.4	100	
	Therapy	77.9	34.6	100	86.5	59.9	100	0.028
	New	74.2	25.3	92.8	73.9	50	100	
	Progressive	74.6	50.8	100	100	45.7	100	
CD30	Control				2	0	3	
	MGUS	1.1	0	3.8	0	0	5.4	
	Complete				0	0	24.5	
	Therapy	0.6	0	68	0	0	12.5	
	New	3.1	0	9.8	3.4	0	37.3	
	Progressive	1	0	43.6	0	0	100	
CD58	Control				96.1	92.2	100	
	MGUS	95.9	94.7	96.4	96.9	92.6	99.2	
	Complete				98.8	83.9	100	
	Therapy	98.4	57.7	100	90.2	82.7	100	
	New	94.1	58.2	100	100	96.5	100	
	Progressive	94.9	59.8	98.2	100	93.5	100	0.005
CD11a	Control				96.6	81.5	98.9	
	MGUS	98.3	97.1	99.3	98.2	89.2	98.8	
	Complete				83.7	64.7	100	
	Therapy	96.4	79.6	100	94.9	93.2	100	
	New	98.6	88.1	100	100	99.7	100	
	Progressive	98.4	20.4	99.3	97	64.7	98.4	
CD117	Control					0		
	MGUS	85.4	15.2	94.7		0		0.005
	Complete					0		
	Therapy	62.6	0	100		0		0.012
	New	21.4	0.3	71.7		0		0.027
	Progressive	49.7	26.2	64.9		0		0.005
CD27	Control				98	97.3	100	
	MGUS	77.7	31.8	100	93.8	83.4	100	0.017
	Complete				97.3	94.2	100	
	Therapy	97.8	0	100	98.2	45.5	100	
	New	80.9	11.6	100	100	28.3	100	
	Progressive	55.4	19.5	100	85.3	72.4	96.5	0.012
CD200	Control				1.3	0	7	
	MGUS	10.6	6.4	42.7	29.7	17.8	65.9	0.035
	Complete				15.5	5.1	100	
	Therapy	53.2	0	88.6	36.3	0	69	
	New	90.4	0.6	99.2	57.5	28.7	69.2	
	Progressive	54.1	17.8	83.4	48.7	11.9	60.7	
CD20	Control					0		
	MGUS	2.4	0	9.2	3.3	0	9.1	
	Complete				4.3	0	26.1	
	Therapy	8.5	0	72.7	9.6	1.9	64.6	
	New	0	0	99.6	0	0	2.7	
	Progressive	0.8	0	4.1	6.6	0	28	0.038

P values referred to intragroup pairwise comparison between CPCs and PPCs are indicated.
Min: minimum; Max: maximum.

Table 5
Levels of Expression of the Studied Markers in all Groups

		MFI Ratio for CPCs			MFI ratio for PPCs			
		Median	Min	Max	Median	Min	Max	*P* value
CD45	Control				12.1	4.7	16.3	
	MGUS	5	4.4	10.5	12	8.4	16.4	0.005
	Complete				11.1	4.3	24.7	
	Therapy	5.2	2.8	25.3	8.1	3.8	23.3	
	New	9.4	3.5	23.5	5.5	1	20.4	
	Progressive	7.6	4.3	9.3	22.3	11	25	0.005
CD19	Control				11	6.2	15.7	
	MGUS		1		9.2	8.6	26.1	0.005
	Complete				8.7	6.7	16.4	
	Therapy	1	1	20.1	8.2	5.2	17.1	
	New	5.3	1	11.6	7.8	1	7.9	
	Progressive		1		9	6.5	16.1	0.005
CD56	Control				16.5	9.4	17.7	
	MGUS	13.1	11	19.3	16.8	8.4	47.2	
	Complete				10.1	1	63.4	
	Therapy	13.9	1	43.4	10.2	1	31.4	
	New	13.3	6.1	19.9	16.7	13	27.3	0.027
	Progressive	15.8	5.9	18	14.4	11.4	19	
CD49d	Control				17.4	11.4	18.2	
	MGUS	14.5	10.1	25.1	19.9	19.1	20.6	
	Complete				21.1	8.5	43.6	
	Therapy	11.8	6.1	39.4	15.3	7	43.4	
	New	11.2	5.5	24.6	13.5	11.1	16.7	0.027
	Progressive	8.2	5	12.5	12.8	6.1	35.1	0.035
CD30	Control				6.5	1	8.2	
	MGUS	9.8	1	10.7	1	1	11.2	
	Complete				1	1	20.7	
	Therapy	4.1	1	39.3	1	1	8.6	
	New	7.3	1	10.9	6	1	12.4	
	Progressive	4.5	1	8.3	1	1	11.9	
CD58	Control				10.2	4	19.5	
	MGUS	7.6	7.2	16.7	11.8	8.5	14.8	
	Complete				12.5	6.6	21.1	
	Therapy	8.9	3.2	30.2	5	3.7	10.5	
	New	6.8	3.7	34.6	11.7	5.3	29.1	
	Progressive	10.3	4	12.2	21.8	7.2	35.1	0.005
CD11a	Control				3.8	3.2	4.6	
	MGUS	4.7	4.3	5.5	4.6	3.7	5.8	
	Complete				4.5	3.3	5.7	
	Therapy	4.6	3.7	18.1	5	3.1	7.6	
	New	6.4	3	15.8	8.1	5.1	17.2	
	Progressive	5.7	2.1	9.2	5.8	4.1	16.5	
CD117	Control					1		
	MGUS	14.4	8.6	14.7		1		0.005
	Complete					1		
	Therapy	7.3	1	23		1		0.012
	New	10.7	6.9	16.3		1		0.027
	Progressive	6	5	11.6		1		0.005
CD27	Control				38.7	19.9	47.3	
	MGUS	37.1	7.9	55.8	46.8	24.5	67.8	0.027
	Complete				42.2	12	55	
	Therapy	29.2	1	62.5	51.6	6	104.2	
	New	11.6	3.6	30.7	21.1	14.5	34.3	
	Progressive	15.7	6.4	44.1	35.8	21	58.4	0.005
CD200	Control				5.2	1	5.8	
	MGUS	7.4	6.3	8.2	8.2	7.7	8.3	0.005
	Complete				8	1.3	12.6	
	Therapy	6.8	1	16.8	8.5	1	12.9	
	New	15.2	7.2	43.4	9.5	9	10	
	Progressive	7.8	4.8	11.6	8.6	5.9	15.4	0.005
CD20	Control					1		
	MGUS	16.9	1	44.2	18.8	1	37.6	
	Complete				6.3	1	36.5	
	Therapy	12.2	1	42.1	14.4	4.4	46.1	
	New	1	1	30.6	1	1	16.9	
	Progressive	16.9	1	32	3.3	1	37.7	

P values referred to intragroup pairwise comparison between CPCs and PPCs are indicated. Min, minimum; Max, maximum.

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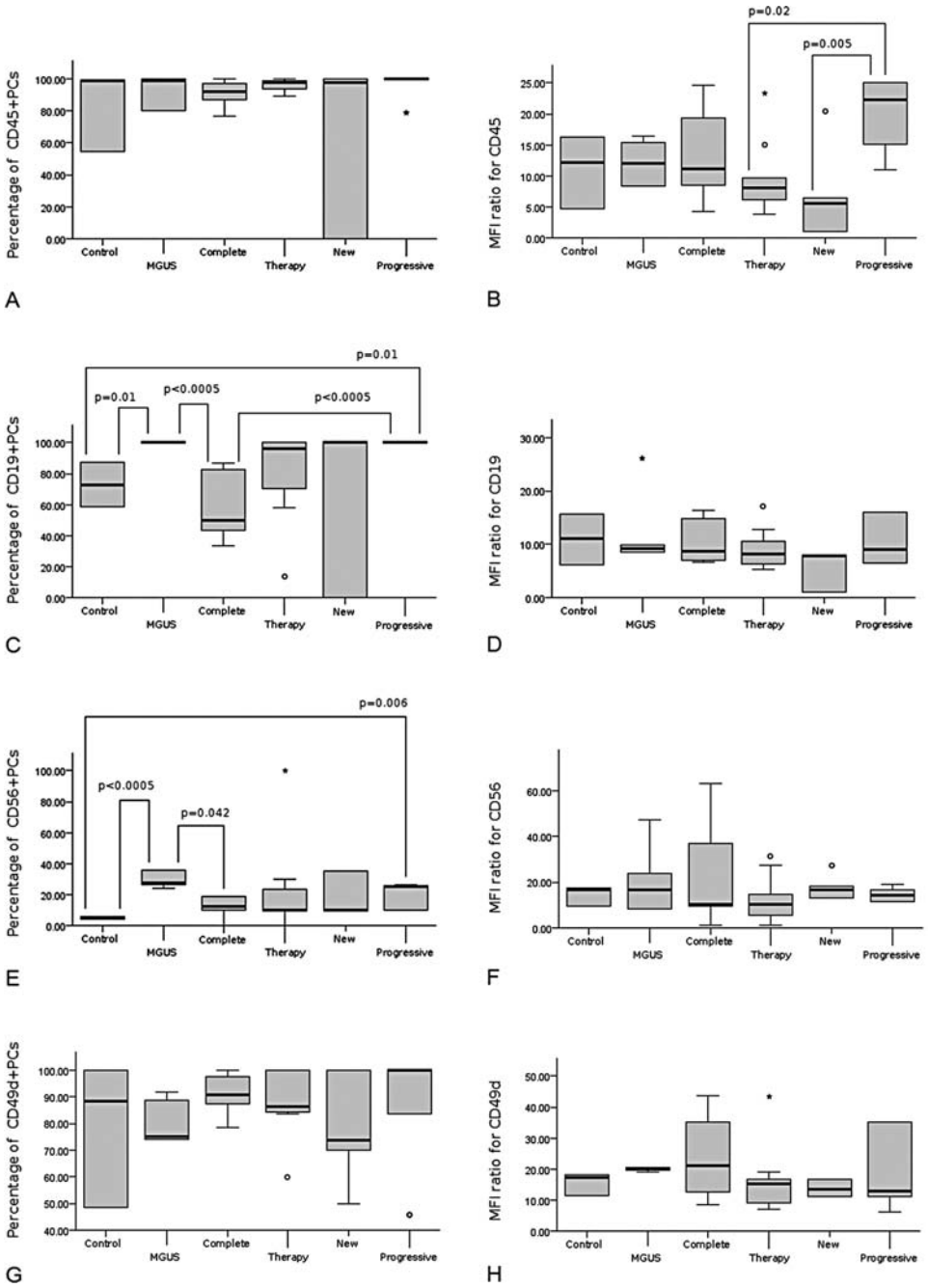

FIG. 1. **A–J**: Immunophenotypic differences in PPCs among groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. The following markers exhibited statistically significant differences as regards the mean percentage of positive cells: CD19 ($P<0.0005$), CD56 ($P<0.0005$), CD58 ($P=0.004$), CD11a ($P=0.01$), CD27 ($P=0.01$), CD200 ($P<0.0005$), and CD20 ($P<0.0005$). Also statistically significant differences as regard MFI ratios were demonstrated: CD45 ($P=0.005$), CD49d ($P=0.02$), CD58 ($P=0.011$), CD11a ($P<0.0005$), CD200 ($P=0.001$), and CD20 ($P=0.008$). *P* values of pairwise comparisons are indicated. CD117 is never expressed on PPCs (data not shown). Levels of expression of CD45 were higher in Progressive than in New and Therapy (A,B). The proportion of CD19+ PCs was lower in Control and Complete than in MGUS and Progressive (C,D). Median percentage of CD56+ PCs was lower in Control versus MGUS and Progressive, and in Complete versus MGUS (E,F). Levels of expression of CD49d did not exhibit statistically significant differences at post hoc analysis (G,H). CD30 did not show variability in expression among groups (I,J). **K–T**: Immunophenotypic differences in PPCs among groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. *P* values of pairwise comparisons are indicated. CD58+ fraction was littler in Therapy than in New and Progressive; also levels of expression of CD58 were lower in Therapy than in Progressive (K,L). CD11a+ fraction was larger in New than in Complete and Progressive; moreover expression of CD11a was less intense in Control than in Progressive and New (M,N). the proportion of CD27+ PCs was lower in Progressive versus Control and New (O,P). CD200 was expressed by a less extended percentage of PCs in Control than in MGUS, New, Progressive and Therapy. MFI ratio for CD200 was lower in Control than in New, Therapy and Progressive (Q,R). Median percentage of CD20+ PCs was higher in Therapy than in Control and New. Intensity of expression of CD20 was reduced in PCs of Control compared with MGUS and Therapy (S,T). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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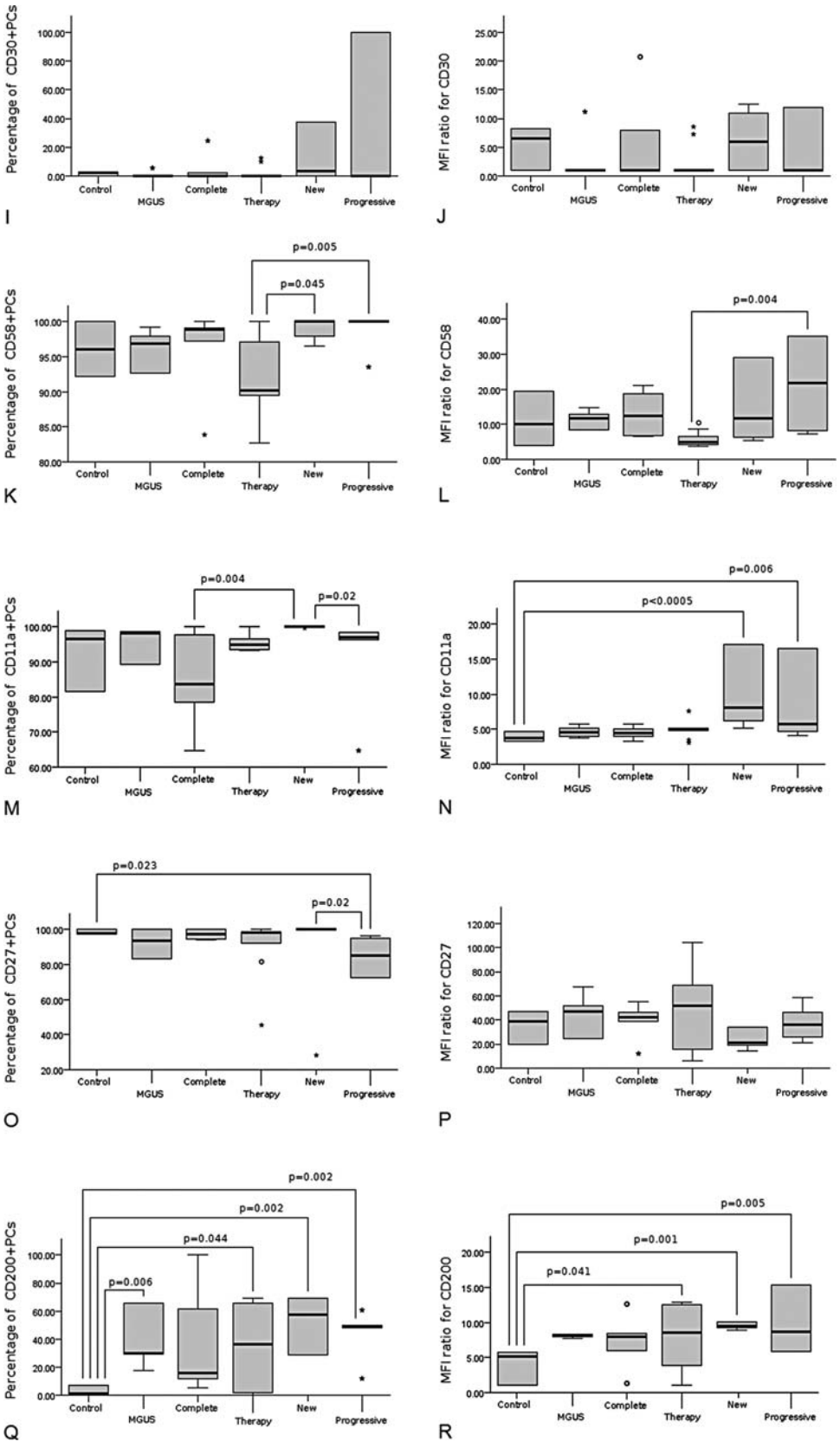

FIG. 1. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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explored immunophenotype of PPCs only in healthy individuals (42,56), while others reunited in the same category PPCs of healthy and pathological subjects (40,45), causing a loss of information related to possible PPC heterogeneity among different categories of patients. Analyzing our data, it can be easily noted that for CD19, CD45, CD27, and CD117 percentages of positive PPCs reflect the ranges found in precedent studies (10,22,23,40–42,56,57); CD20 was never expressed on PCs in Control group, but the range of expression was broad on PPCs in all other groups. CD200 was expressed on both normal and neoplastic PCs, as previously reported (10,58). CD58, CD49d, and CD11a were never completely absent on PPCs and CPCs, while frequency of CD30+ cells was modest in both plasmacellular compartments in all groups (36). In this study, range of expression for **CD56** (NCAM – Neural Cell Adhesion Molecule) on PPCs essentially did not depart from those described elsewhere (40,42,56), except for an outlier identified in Therapy; however expression close or corresponding to 100% for this antigen has occasionally been reported (32,56). The proportion of CD56+ PCs is constantly described to be low in BM of healthy controls (10,57), however CD56+ PCs may be artificially low in MFC analysis; acting as an anchor, CD56 could make PCs more resistant to BM aspiration (42). Focusing on ranges of expression on PPCs, we observed that CD56+ fraction is reduced in Control than in MGUS and Progressive, and in Complete than in MGUS. This is openly conflicting with report by Pérez-Andrés et al. who described no phenotypic differences for CD56 between PCs from healthy individuals and PPCs from MGUS subjects (45). This discrepancy may be explained considering differences in gating strategies, and in the use of monoclonal antibody clones or fluorochromes between the cited study and ours. As expected, CD56+ CPCs were more numerous than CD56+ PPCs in New, Progressive, and Therapy; the same type of relation was maintained in comparisons with Control and Complete PCs. However, in New, MFIs ratio of PPCs was higher than that of CPCs; to the best of our knowledge, this observation has never been described since the introduction of >4 color FC in analysis of MM patients. On the basis of these results, the expression of CD56 on PPCs should be further elucidated. **CD45** on PPCs is reported to be expressed heterogeneously (10,22,23,40,57); in our study it was effective in distinguishing PPCs from CPCs in MGUS, Progressive and Therapy. Expression of CD45 was more intense on PPCs in Progressive than on PPCs in New and Therapy. Considering that CD45 expression characterizes proliferating compartment of normal, reactive and malignant PCs (13,22), this increased level of expression on Progressive PPCs may depend on the proliferative BM microenvironment to which all PCs of relapsing subjects are exposed (24). **CD19** is downregulated in PCs maturation, and CD19-CD56+ PCs are believed to represent long lived terminal stage PPCs (22,40). CD19 was persistently negative in MGUS and Progressive CPCs. This pattern of expression has already been described in literature (22,40), although cases of CD19+ MGUS cells have been reported (22). Clearly, PCs from Control and Complete exhibited a reduced CD19+ fraction compared to PPCs from MGUS and Progressive. Exact mechanism ruling expression in MGUS and Progressive subjects remains to be explored. **CD27** is strongly expressed on PPCs (9,10,56), but CPCs are reported to be weak or negative (9,10,57); in our study it showed no differences in expression comparing CPCs with Control and Complete PCs, contradicting previous data (9), probably because of discrepancies in methods or the small sample size. We evidenced that CD27+ Progressive PPCs were less numerous than CD27+ PPCs of New and Control. Since this antigen is involved in apoptosis induced by CD27–CD70 interactions (59), it would be interesting to evaluate if other molecules required for programmed cell death show variable expression in PPCs of Progressive patients. **CD117** was never present on normal PCs, thus being highly effective in distinguishing PPCs from CPCs (when present on CPCs). The data about **CD20** in PCs of healthy people are conflicting. Reported ranges vary from a minimum of 0 to maximum of 91% (10,22,40,56,57), but it is universally accepted that low CD20 expression is associated to PC maturity. Expression of CD20 on MM cells is reported to range from 2 to 90% (42). CD20 was a poor antigen in distinguishing CPCs from PPCs, as previously described (56), except in Progressive group, in which proportion of CD20+ PPCs was higher than proportion of CD20+ CPCs. Expression of CD20 on CPCs is associated t(11;14) (Refs. 22) and (23)); since cytogenetic analysis was not performed, we can not say if t(11;14) is present in Progressive CPCs. In

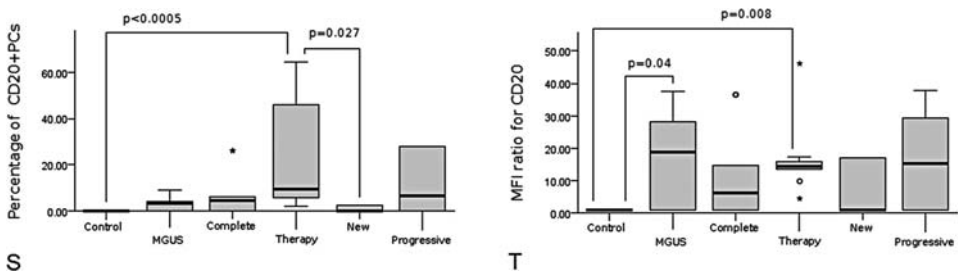

FIG. 1. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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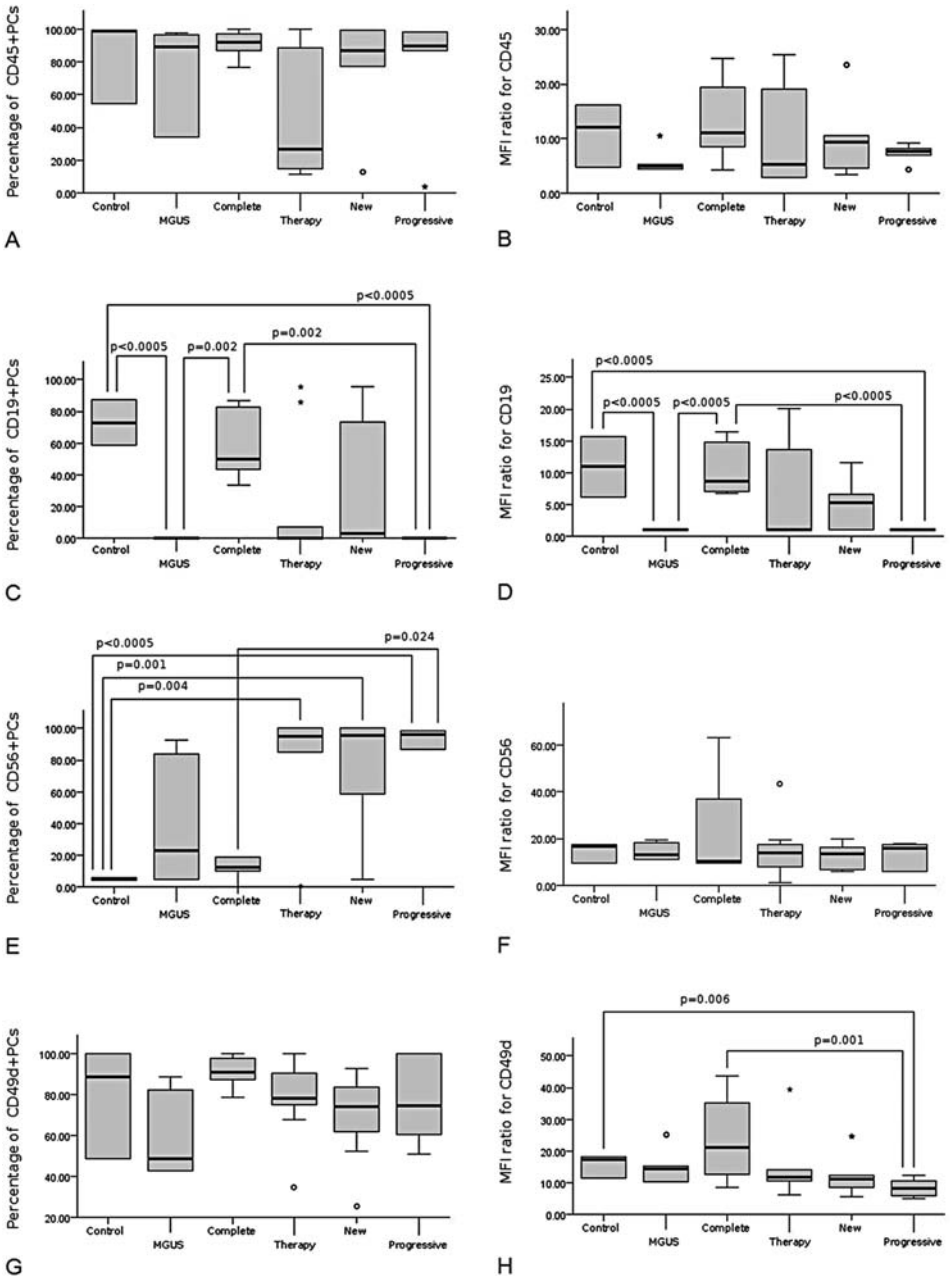

FIG. 2. **A–L**: Results of comparisons among PCs of Control and Complete and CPCs of MGUS and MM groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. Kruskal-wallis test revealed that the following markers showed statistically significant differences as regards the median percentage of positive cells among groups: CD19 ($P < 0.0005$), CD56 ($P < 0.0005$), CD117 ($P < 0.0005$), CD200 ($P < 0.0005$), and CD20 ($P = 0.004$). Moreover, statistically significant differences in intensity of expression emerged among groups for: CD19 ($P < 0.0005$), CD49d ($P < 0.0005$), CD11a ($P = 0.01$), CD117 ($P < 0.0005$), CD200 ($P < 0.0005$), and CD20 ($P = 0.013$). *P* values of pairwise comparisons are indicated. CD45 did not show variability in expression among groups (A,B). Median percentage of CD19+ PCs in Control was higher than in MGUS and Progressive; also CD19+ fraction of PCs was larger in Complete than in Progressive and MGUS. Levels of expression of CD19 were higher in Control and Complete than in MGUS and Progressive (C,D). Proportion of CD56+ PCs was lower in Complete than in Progressive, and in Control than in New, Therapy, and Progressive (E,F). Levels of expression of CD49d were higher in Complete and Control than in Progressive (G,H). CD30 did not show variability in expression among groups (I,J). CD58 did not exhibit any statistical significant difference (K,L). **M–V**: Results of comparisons among PCs of Control and Complete and CPCs of MGUS and MM groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. *P* values of pairwise comparisons are indicated. Intensity of expression of CD11a was lower in Control versus Progressive and New (M,N). Since PPCs did not express CD117, the difference of Control and Complete versus all other groups was extremely significant, except versus New ($P = 0.16$). MFI ratio measured for CD117+ cells was different comparing Control and Complete versus New, MGUS and Therapy (O,P). CD27 was homogeneous in its expression comparing all subjects (Q,R). CD200+ fraction is less extended in Control than in CPCs of New, Therapy and Progressive. Levels of expression of CD200 were higher in New versus Control (S,T). CD20+ proportion of PCs was lower in Control than in Therapy. Intensity of expression of CD20 was reduced in Control versus MGUS (U,V). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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addition, deeper molecular studies are necessary to explain the reasons why MFI ratio was reduced in Control versus MGUS CPCs and PPCs. Therapy PPCs showed upregulation of CD20 compared with Control and New PPCs, and also proportion of CD20+ CPCs in Therapy is larger compared with Control PCs. Recently a study described the transcriptional effects of bortezomib on human myeloma cell lines, showing a

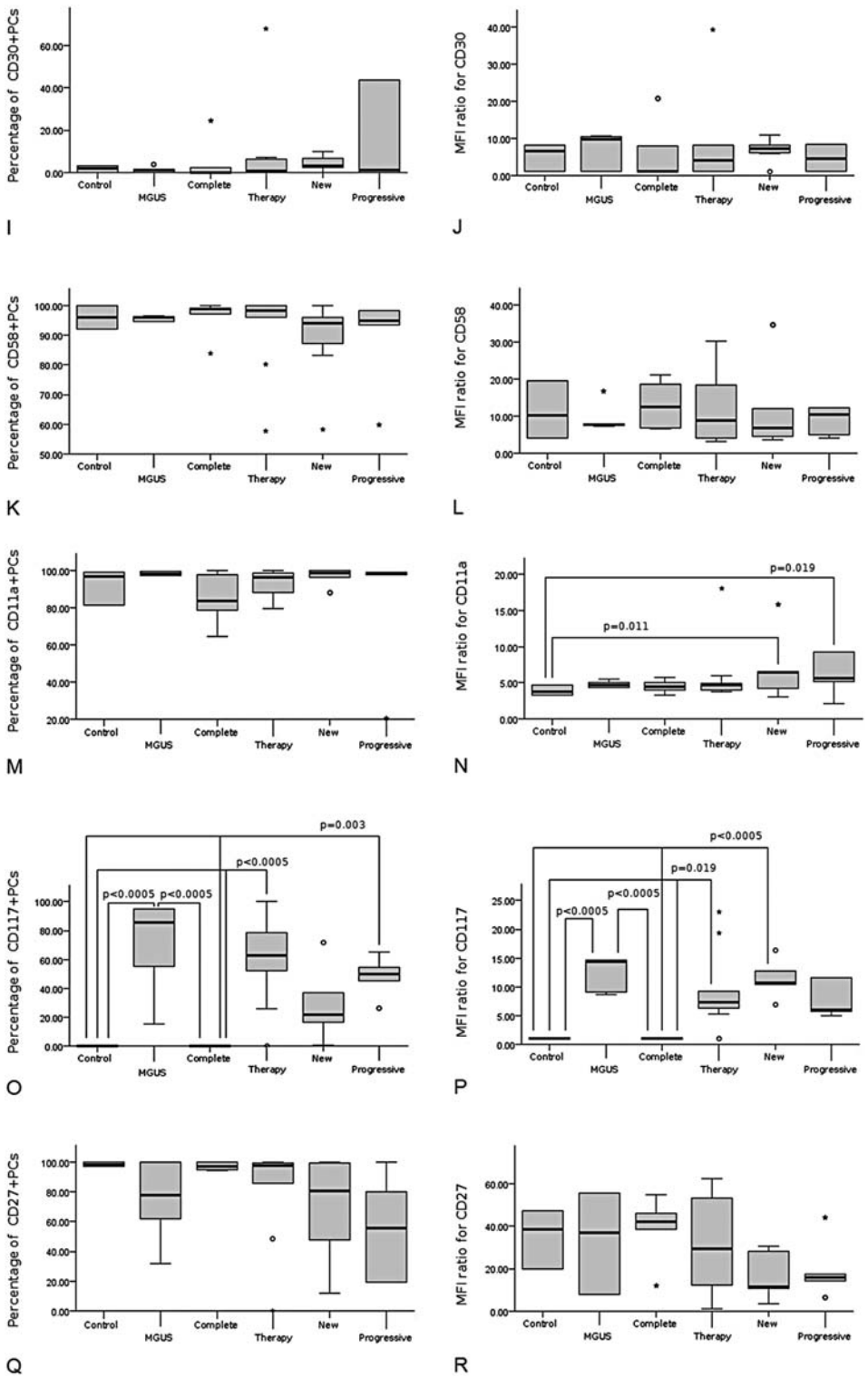

FIG. 2. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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downregulation of many antigens but no effect on CD20 expression (60). Since in this study 70% of Therapy patients are currently treated with bortezomib containing regimens, we hypotize that instead bortezomib could directly influence expression of CD20 in PCs, although confirmation with larger groups of patients is necessary to confirm or reject this hypothesis. **CD200** is a cell surface glycoprotein expressed on normal B-cells and some T-cell subsets; the expression of CD200 showed to be positive on MM PCs up to 78% (27,61). CD200 on PPCs has been poorly investigated, and no clear range of expression has been defined; however our data match with both papers reporting its absence (26,56) and those indicating positivity (10,58). Evaluating its utility in CPC detection, we observed that in MGUS CD200 was expressed by a higher percentage of PPCs vs.CPCs. In addition, expression was more intense on MGUS and Progressive PPCs vs. MGUS and Progressive CPCs. Defining normal phenotype, Control PCs showed less extended fraction of CD200+ cells vs. MGUS, New, Progressive, and Therapy PPCs, and a dimmer expression vs. MM groups. Comparing Control and Complete PCs with CPCs, once again Control displayed a smaller proportion of CD200+ cells vs. Therapy, New, and Progressive. CD200+ positive cells present reduced immunogenicity compared with normal lymphocytes (27) and absence of CD200 correlates with a better prognosis compared to its presence in MM (12); on this basis, we would have expected a higher expression of CD200 in Progressive patients vs. Complete. Deeper investigation on relapse molecular mechanisms is necessary before excluding a role of CD200 in progression of MM. **CD11a**, the integrin α subunit of LFA-1, has been reported positive as well as negative in literature on MGUS and MM samples (28,30). The ranges of expression on CPCs are really different from one paper to another, ranging from 0% to 100% in newly diagnosed or remitting subjects, and from 0 to ~80% in relapsing subjects (32), or reported as <30% for CD45+ cells and ≪10% for CD45- cells (34). On PPCs, it has been reported to range from 20 to 100% (32). In our study, CD11a+ proportion of CPCs showed no differences compared to CD11a+ Control and Complete PCs, while levels of expression of CD11a were higher on both CPCs and PPCs of New and Progressive vs.Control PCs; these results diverges from a precedent report demonstrating a downregulation of CD11a in patients in chronic phase, but in that case results might be compromised by the inclusion of newly diagnosed and remitting subjects in the same group (32). Examining PPCs, CD11a+ fraction was expanded in New vs.Complete and Progressive. **CD58**, a CD2 receptor, was previously indicated as present occasionally on PPCs and never expressed on MGUS CPCs (28); in our study, it is positive in a fraction of PPCs and MGUS CPCs close to 100%. This discrepancy may be attributed to differences regarding methods (immunofluorescence microscopy vs. MFC) and choose of control samples. CD58 was more expressed on PPCs than on CPCs in Progressive (considering both proportion of CD58+ cells and MFI ratio). Therapy PPCs showed downregulation of CD58 vs.New and Progressive. These data suggest that CD58 should be considered in further studies to assess its ability in predicting response to therapy or progression of disease when evaluated on non neoplastic population. We did not notice any difference comparing Control and Complete with CPCs, contrary to previous data (28). **CD49d**, the integrin α subunit of VLA-4, has been found strongly expressed by MM PCs (36), while mean percentage of positive normal PCs has been reported ~75%

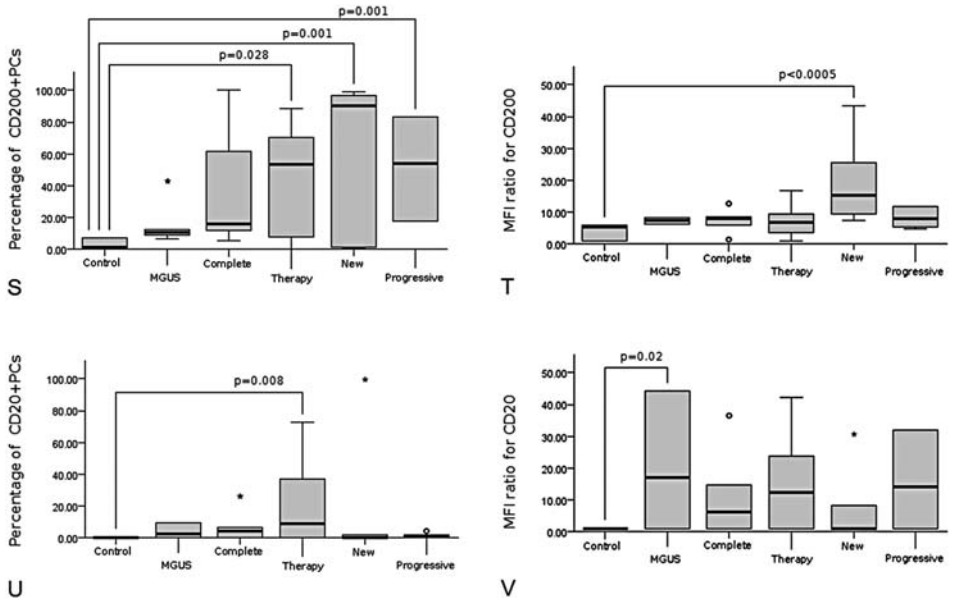

FIG. 2. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Table 6
Results of Multinomial Logistic Regression Analysis

		Univariate			Multivariate		
Independent variable	PCs	Exp (B)	95% CI	P value	Exp (B)	95% CI	P value
MFI ratio for CD45	PPCs	1.03	0.88–1.20	0.722[a]			
		1.06	0.91–1.23	0.472[b]			
		0.93	0.79–1.11	0.438[c]			
		0.81	0.63–1.03	0.086[d]			
		1.25	1.06–1.50	0.011[e]	1.23	1.04–1.46	0.015
Percentage of CD19+ cells	CPCs	111.1	111.1–111.1	Na[f]			
		111.6	108.9–114.4	<0.0005[g]	144.9	139.8–150.3	<0.0005
		1		1.0[h]			
MFI ratio for CD19	CPCs	67.6	67.6–67.6	Na[f]			
		64.6	54.9–76.0	<0.0005[g]	61.5	49.6–76.2	<0.0005
		1		1.0[h]			
	PPCs	1.04	0.87–1.22	0.670[a]			
		0.93	0.77–1.14	0.502[b]			
		0.88	0.71–1.11	0.298[c]			
		0.57	0.36–0.89	0.016[d]	0.53	0.26–1.08	0.082
		0.95	0.78–1.15	0.606[e]			
Percentage of CD56+ cells	PPCs	1.51	1.16–1.96	0.002[a]	1.71	1.19–2.42	0.003
		1.33	1.03–1.71	0.029[b]	1.53	1.08–2.16	0.016
		1.48	1.14–1.92	0.004[c]	1.66	1.17–2.36	0.005
		1.43	1.10–1.87	0.008[d]	1.61	1.13–2.29	0.009
		1.46	1.13–1.90	0.004[e]	1.54	1.07–2.21	0.019
MFI ratio for CD49d	CPCs	0.98	0.86–1.09	0.684[f]			
		0.94	0.82–1.08	0.394[g]			
		0.68	0.51–0.92	0.012[h]	0.62	0.38–0.99	0.046
MFI ratio for CD58	PPCs	0.99	0.86–1.13	0.882[a]			
		1.03	0.90–1.16	0.695[b]			
		0.64	0.45–0.93	0.019[c]	0.55	0.37–0.84	0.025
		1.06	0.93–1.22	0.373[d]			
		1.13	0.99–1.27	0.057[e]			
MFI ratio for CD11a	PPCs	4.29	1.10–16.7	0.036[a]	3.19	0.63–15.9	0.158
		4.01	1.03–15.6	0.045[b]	2.41	0.50–11.6	0.273
		5.51	1.38–21.9	0.016[c]	4.82	1.20–19.3	0.027
		10.8	2.64–44.6	0.001[d]	13.7	1.95–95.9	0.008
		10.1	2.48–41.5	0.001[e]	14.1	2.74–72.4	0.02
Percentage of CD117+ cells	CPCs	0.99	0.95–1.02	0.448[f]			
		0.93	0.88–0.97	0.003[g]	0.94	0.89–0.99	0.023
		0.97	0.93–1.01	0.095[h]			
MFI ratio for CD117	CPCs	0.84	0.66–1.06	0.143[f]			
		0.95	0.77–1.17	0.625[g]			
		0.71	0.53–0.94	0.017[h]	0.65	0.42–1.01	0.054
Percentage of CD200+ cells	CPCs	1.04	0.99–1.07	0.057[f]			
		1.05	1.01–1.09	0.008[g]	1.06	1.01–1.11	0.012
		1.04	1.01–1.08	0.032[h]	1.04	0.99–1.09	0.084
	PPCs	1.31	1.07–1.61	0.009[a]	1.36	0.95–1.95	0.089
		1.3	1.07–1.60	0.010[b]	1.36	0.95–1.94	0.089
		1.3	1.06–1.60	0.011[c]	1.36	0.95–1.94	0.091
		1.34	1.09–1.64	0.005[b]	1.38	0.97–1.97	0.073
		1.32	1.08–1.62	0.007[e]	1.63	0.95–2.80	0.075
MFI ratio for CD200	CPCs	1.01	0.78–1.30	0.957[f]			
		1.31	1.02–1.68	0.034[g]	1.38	1.02–1.88	0.038
		1.06	0.83–1.35	0.656[h]			
	PPCs	1.56	1.12–2.16	0.008[a]	2.02	1.19–3.41	0.008
		1.45	1.06–1.98	0.020[b]	1.35	0.88–2.07	0.172
		1.49	1.08–2.07	0.016[c]	1.73	1.11–2.70	0.016
		1.8	1.12–2.65	0.003[d]	2.59	1.25–5.35	0.01
		1.86	1.30–2.65	0.001[e]	2.23	1.28–3.89	0.005

[a]Control vs. MGUS.
[b]Control vs. Complete.
[c]Control vs. Therapy.
[d]Control vs. New.
[e]Control vs. Progressive.
[f]MGUS vs. Therapy.
[g]MGUS vs. New.
[h]MGUS vs. Progressive.

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(29). In our study, CD49d showed a more pronounced expression on PPCs than on CPCs in all groups (fraction of positive cells for MGUS and Therapy, MFI ratio for New and Progressive), thus beeing highly useful to refine CPC population when combined with common used marker. Intensity of expression was higher in Control and Complete PCs vs.Progressive CPCs, so it would be intriguing to verify if this marker has a role in drug resistance during progression of disease. As we could not identify a univocous pattern of expression of these three adhesion involved markers, regulatory pathways determining their expression should be explored individually. Continuous efforts are made in MM to improve the sensitivity of immunophenotypic detection of PCs. On the basis our results, we suppose that in future panels of markers specific for the clinical question could be used, with the addition of appropriate MFC markers to the backbone ones (CD38, CD138, CD19, CD45, and CD56); the evaluation of the disease should involve PPCs, in order to follow progressions and explore the opportunity to obtain predictive information. CD49d should be universally present to distinguish CPCs from PPCs; for MGUS diagnosis and monitoring, CD20 and CD200 are suggested to be added; newly diagnosed patients may benefit the addition of CD11a; assessment of disease progression or response to therapy would be more accurate by the introduction of CD27, CD58, CD11a, and CD20. The prognostic value of specific antigenic profiles (CD19, CD117, and CD27) has already been assessed; positive staining for CD19 and absence of CD117 detected on clonal PCs were associated with significantly shorter progression free survival and OS (overall survival) rates of MM patients. Moreover, OS rates were higher in patients with CD27+ MM than in those who were CD27- (57,62,63). The absence (or presence) of CD200 expression in MM cells is predictive for event-free survival independently of ISS stage or β-2-microglobulin serum levels (12). For CD45 and CD56 results from precedent studies are contradictory, and require further investigation (24,57). No information is available about the prognostic value of the other markers used in this study, or about their role in predict response to therapy and disease progression, and their utility in distinguishing disease stages without considering other clinical variables. To see if a correlation exists between the expression of each marker and the attribution of the patients to their own groups, we performed a multinomial logistic regression analysis; results surprisingly revealed that the most abundant associations regarded PPCs, with percentage of cells positive for CD200 and CD56, and levels of expression of CD45, CD19, CD58, CD11a, and CD200 showing statistical significance. In addition, fractions of CPCs positive for CD19 and CD200, and intensity of expression of CD19, CD49d, CD117, and CD200 on CPCs were significant. But when the model was elaborated again including significant prognostic variables (37,38,43), percentage of CD200+ PPCs and levels of expression of CD117 on CPCs and CD19 on PPCs loosed their association, as did levels of expression of CD11a comparing Control with Complete and MGUS PPCs, MFI ratio for CD200 analyzing Control vs. Complete PCs, and percentage of CD200+ CPCs comparing MGUS vs. Progressive. This is not surprising, since other works have already demonstrated relations between variation in expression of MFC markers and BM features (64). Our data comfort the hypothesis that, in assessing the predictive/prognostic values of MFC markers and their utility in following disease steps, immunological-biochemical-hematological profile must not be ignored since it is the "mirror" of the altered BM microenvironment influencing/being influenced by PC phenotype (37,64). However CD19 and CD49d on CPCs, and CD45, CD58 and CD56 on PPCs maintained their explicative power, so they are good candidates for further studies, especially CD19, which was considered a "reliable" antigen in a report exploring relationship between MFC and histological results (56). In conclusion, we have provided a feasible start point to put in order the ranges of expression on PPCs in healthy and myeloma subjects; we propose a new approach based on analysis of PPCs to monitor the stages of the disease. However, the study size is small, and we could not provide a prospective cohort to examine fluctuations in expression of MFC markers related to time and disease progression, just as we could not define a threshold of expression for each marker that allows to clearly attribute each subject to his own group. Confirmation by larger and deeper independent studies, as well as by the elaboration of an adequate Cox proportional-hazard model, is indicated.

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Supplementary Information

Table SI 1. Antibodies used in this study

Antigen	Manufacturer	Catalogue #	Clone	Fluorochrome	Isotype
CD11a	Beckman Coulter	IM0860U	25.3	FITC	IgG1 Mouse
CD19	Beckman Coulter	A86355	J3-119	Pacific Blue	IgG1 Mouse
CD20	Beckman Coulter	IM1455U	B9E9 (HRC20)	FITC	IgG2a Mouse
CD27	Beckman Coulter	B12701	1A4CD27	APC-Alexa Fluor 750	IgG1 Mouse
CD30	BD Pharmingen™	555829	BerH8	FITC	IgG1 Mouse
CD34	Beckman Coulter	A89309	581	APC-Alexa Fluor 750	IgG1 Mouse
CD38	Beckman Coulter	A70205	LS198-4-3	PC5.5	IgG1 Mouse
CD45	Beckman Coulter	A96416	J.33	Krome Orange	IgG1 Mouse
CD49d	BD Pharmingen™	555503	9F10	PE	IgG1 Mouse
CD56	Beckman Coulter	IM2073U	N901 (NKH-1)	PE	IgG1 Mouse
CD56	Beckman Coulter	A51078	N901 (NKH-1)	PC7	IgG1 Mouse
CD58	Beckman Coulter	IM1430	AICD58	PE	IgG2a Mouse
CD117	Beckman Coulter	IM3698	104D2D1	PC7	IgG1 Mouse
CD138	Beckman Coulter	A87787	B-A38	APC	IgG1 Mouse
CD200	BD Pharmingen™	561762	MRC OX-104	PE	IgG1 Mouse
κ CHAIN	Beckman Coulter	A64828	Polyclonal	FITC	$F(ab')_2$ Rabbit
λ CHAIN	Beckman Coulter	A64827	Polyclonal	PE	$F(ab')_2$ Rabbit

Catalogue #, catalogue number; FITC, Fluorescein Isothiocyanate; PC5.5, R-Phycoerythrin-Cyanin 5.5; PE, R-Phycoerythrin; PC7, R-Phycoerythrin-Cyanin 7; APC, Allophycocyanin. Beckman Coulter (Miami, FL, USA), BD Pharmingen™(San Jose, CA, USA).

Table SI 2. Characteristics of the instrument.

Model (manufacturer)	Lasers	Detector Filters
Navios (Beckman Coulter) 10 colors, 3 lasers (5+3+2 configuration)	Blue Solid State Diode: 488nm, 22mW laser output	Forward Scatter: 488/10 Blue Laser: 525/40, 575/30, 620/30, 675/20, 695/30, 755LP
	Red Solid State Diode: 638nm, 25mW laser output	Red Laser: 660/20, 725/20, 755 LP
	Violet Solid State Diode: 405nm, 40mW laser output	Violet Laser: 450/50, 550/40

Beckman Coulter (Miami, FL, USA)

4. Utility of CD54, CD229 and CD319 for the identification of normal and aberrant plasma cells.

Manuscript in preparation

TITLE

Utility of CD54, CD229 and CD319 for the identification of normal and aberrant plasma cells.

RUNNING TITLE

CD54, CD229 and CD319 as plasma cell markers

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Keywords: multiple myeloma, multiparameter flow cytometry, new markers, plasma cells, minimal residual disease, immunophenotyping, EuroFlow.

Abstract

Background: Multiparameter flow cytometry (MFC) characterization of aberrant plasma cells (aPC), in contrast to normal PC (nPC), is a useful tool to support diagnosis, prognostication and monitoring of plasma cell dyscrasias (PCD). The current list of MFC markers for PCD evaluation remains limited and has not augmented significantly in the past years. Moreover, therapies based on antibodies against CD38 or CD138 compromise the utility of these molecules for PCD follow-up. Revision of literature resulted in a list of candidates to be evaluated as alternatives to classical markers: CD54, CD229, and CD319. Methods: we characterized the expression of CD229, CD54 and CD319 on PC and their utility in MFC evaluation of PCD, analyzing bone marrow (BM) samples from healthy controls (HC) and PCD patients, and PB specimens from HC. Results: In both BM and PB PC, the expression of the studied markers is better preserved compared to CD138. Setting the initial gate in the bivariate dot plots CD319, CD229 or CD54 vs CD38 allowed the inclusion of 100% PC. When the markers were combined with SSC and CD138, CD229 allowed the best identification of PC; in turn CD54 and CD319 showed to be of limited utility, because the expression pattern of myeloid populations may overlap with that of PC. Conclusions: We have demonstrated that CD229 could be considered as a marker able to bypass the need of CD38 in PC detection and needs to be tested in clinical practice, so more efforts should be performed in this sense in future.

Introduction

Neoplastic plasma cell disorders (PCD) are a heterogeneous group of disorders characterized by the presence and accumulation of abnormal clonal plasma cells (aPC), typically associated with presence of monoclonal immunoglobulin (M protein) in the serum and urine. The clinical spectrum of PCD ranges from asymptomatic (monoclonal gammopathy of uncertain significance -MGUS- and smoldering multiple myeloma –SMM-) to symptomatic multiple myeloma -MM- and plasma cell leukemia (PCL) [1]. Multiparameter flow cytometry (MFC) quantification and characterization of aPC (in contrast to normal/reactive polyclonal PC -nPC-) is recognized as a very useful tool to support diagnosis, prognostication and monitoring of PCD [2-6]. The current list of useful MFC markers for PCD evaluation remains limited if compared to other clonal hematopoietic disorders and it has not augmented significantly in the past years [6-9]. More recently, important many efforts have been made in order to standardize methodological grounds and clearly define the PC staining profiles obtained with the use of distinct antibody (Ab) reagents combinations [8], nonetheless, evaluation of new candidate markers with potentially contribution for PC identification and nPC vs cPC discrimination has not been deeply explored [9]. Moreover, the introduction of new therapeutic approaches targeting classical PC surface molecules with

monoclonal antibodies (i.e. therapies directed against CD38 or CD138) [10, 11], impacts the utility of these molecules for MM after-treatment follow-up. Similarly, sample "age" may also compromise the detection of markers like CD138 [12, 13]. The need of new useful markers for PC detection and discrimination of aPC from nPC becomes evident.

Critical revision of the literature resulted in a list of candidate markers to be evaluated: CD54, CD229, and CD319. Their expression profile on malignant PC has been previously described [14-16]; all of them have also been investigated as therapeutic targets for multiple myeloma patients[14, 15, 17]. CD229 and CD319 from SLAM family are self-ligand receptors [18], while immunoglobulin superfamily adhesion molecule CD54 has LFA-1 and Mac-1 as physiological ligands [19]. Despite their presence on MM plasma cell surface documented in different experimental settings, none of the cited markers has been evaluated, to the best of our knowledge, as alternative candidate to antigens currently used in MFC detection and evaluation of PC [20]. We characterized the expression of these markers in PC and evaluated their utility as complementary/alternative candidates for MFC evaluation of PCD.

Materials and Methods

As indicated in Table 1, a total of 51 patients -48 BM and 3 extraosseous (extramedullary) plasmacytoma (EMP) samples- and 10 healthy controls - 5 BM, and 5 peripheral blood (PB) samples- were included in this study, after obtaining informed consent according to local ethical committees, in line with Declaration of Helsinki Protocol. BM and EMP samples were obtained from 32 male and 24 female patients [median age 59 (23-79) years]. In turn, PB samples were obtained from 3 male and 2 female healthy donors [median age 41 (37-49) years].

All samples in this study were stained following EuroFlow sample processing Standard Operative Procedures (SOPs) for diagnostic EuroFlow panels [21], both for surface membrane and intracellular markers, with combinations of antibodies indicated in Supplementary Table I. Clones and manufacturer of antibodies are also indicated in Supplementary Table I. We acquired a median of 270,025 events (range 34,151 - 1,629,575) in FACSCanto II instrument (BD Biosciences, San Jose, CA, USA), set-up and monitored according to EuroFlow SOP [21]. Data were analyzed using Infinicyt software v1.8 (Cytognos, Salamanca, Spain). Expression levels are reported as median fluorescence intensity values (MFI; arbitrary units). Statistical analysis was performed with IBM SPSS Software v22 (IBM Corp., Armonk, NY, USA). Mann–Whitney U test was used to assess significance of comparisons between two groups. Kruskal Wallis test with multiple comparisons was used to assess the statistical significance ($p<0.05$) of the differences observed in multiple group comparisons.

Results

In BM samples both CD319 and CD54 were positive virtually on total PC (tPC). CD229 also exhibited 100% positivity on tPC in all but 3 cases (1 MM sample at diagnosis and 2 MRD), on which we observed an heterogeneous expression (from negative to positive) pattern. Negative population included a median 10.9% (range 1.7-26.8) of aPC, and 4.1% (range 4.1-16.3) of nPC. Of note, these samples were stained using a different fluorochrome (i.e. PerCP-Cy5.5), less sensitive compared with APC. In addition, they followed a different sample processing, using a cytoplasmic staining protocol for light chain evaluation. In EMP samples, the studied markers always exhibited 100% positivity. In PB, only CD229 maintained full positivity on tPC. CD319 and CD54 showed a heterogeneous expression ranging from negative to positive in 1/5 (14.3% of negative PC) and 4/5 cases -median 17.4% (14.3%-33.3%) of negative PC-, respectively (Table 1). In both BM and PB, we noticed that the expression of the studied markers is better preserved on PC compared to CD138 expression (Table 1).

In order to detect PC with the evaluated markers, the most useful combinations as initial gate involved the use of CD319, CD229 or CD54 vs CD38. In all analyzed samples, these combinations allowed the inclusion of 100% of both aPC and nPC present as identified with the initial gate set in the classical bivariate dot plot CD38 vs CD138 (Figure 1A-B). Other combinations involving tested markers (i.e vs SSC or CD138) were less powerful, because of a lower intensity observed for them in PB PCs or because the expression pattern in other myeloid populations may overlap with that of the PC, lowering their resolution power, as happened for CD54 and CD319 (Figure 2A-B). More specifically, comparing the efficiency of these markers combined with SSC and CD138 in PC detection, CD229 allowed the best identification of PC in all cases in comparison with the other two markers. In fact, CD229 was expressed simultaneously also on plasmacytoid dendritic cells (pDC) (Figure 2C), whose overlap with PC detection may be overcome using light scatter parameters and other markers present in the panel. In turn CD54 and CD319 vs SSC and/or CD138 showed to be of limited utility to detect PC, especially in case in cases exhibiting compromised expression of CD138. This could be easily explained considering that CD54 was usually positive on monocytes with an heterogeneous pattern (from -/+) (Figure 2A), and both BM and PB staining profiles for CD319 caused PC to be poorly resolved from monocytes (Figure 2B). A different CD319 conjugate, using a brighter fluorochrome (PE; panel 2), did not qualitatively impact the expression pattern described (data not shown). To verify if these three markers can be used for a better separation of aPC from nPC, we compared levels of expression measured on both nPC and aPC for all the samples groups. At the same time, we chose to look for variation in expression of CD54, CD319 and CD54 on PC detected in PB vs BM of healthy and PCD subjects. Results are summarized in Figure 3A-C; in brief in HC, CD54, CD319 and CD229 were significantly downregulated on PB PC compared with BM PC. Additionally, levels of expression

of CD54 and CD319 were lower on aPC vs nPC, although these differences did not reach statistical significance. No difference between aPC/nPC was reported for CD229 in samples stained with panel 1 (Figure 3C) or panel 3 (data not shown). Since CD229 emerged as a reliable and promising identification marker, we explored its expression on other cell types in BM and PB samples from HC (Figure 4A-D). Our objective was to identify other cell types contaminating initial gates in bivariate dot plots CD229vsSSC and CD229vsCD138. In PB and BM, CD229 was expressed on NK-, B- and T-cells (no significant differences among T or B subsets to be shown). Both in PB and BM samples, plasmacytoid dendritic cells (pDC) were always positive whereas neutrophils, eosinophils, monocytes (including CD16+ monocytes/macrophages) showed persistently negativity. BM B-cells showed a spread pattern of expression, ranging from mostly negative $CD34^{+}CD19^{+}$ cells, to immature and naïve B-cells showing an extended amount of positive cells (figure 4A).

Discussion

MFC enumeration of BM PC and particularly the discrimination between normal and abnormal plasma cell populations in patients with PCD proven to be of utility in diagnosis, prognostic stratification and monitoring of MRD levels [2-6]. Accuracy in PC identification and discrimination of normal/reactive vs abnormal/clonal is therefore crucial. No specific PC markers have been described so far, perhaps with the exception of CD138, so MFC detection of PC heavily relies on a relative small number of antigens for which PC show a characteristic expression pattern, just like CD38, CD138, CD19 and CD45 [20], a gating strategy and an experienced operator. Despite this, limited progress have been made in recognition of new markers for PC identification in the last 10 years. More recently, therapeutic antibodies against a variety of PC surface molecules, such as CD38 and CD138, have been developed and are currently under evaluation for treatment of MM patients [10, 11, 22]; the effect of these treatments on MFC staining has not been deeply explored yet, but based on experienced accumulated in similar situations, usage of these therapies will compromise utility of these monoclonal antibodies used as therapeutic agents, thus limiting their value for MFC PC detection, and probably limiting the resolution power of current combination [22, 23]. Moreover, not all antigens used in PC identification and characterization are stable: it is the case of CD138, whose time- and apoptosis-dependent loss from PC surface has been previously demonstrated [12-13]. Although the importance of introduction of new markers in MFC panels has already been assessed [9], many efforts have been made in the identification of therapeutic targets or prognostic combinations but not in detection markers for aPC vs nPC discrimination. Here we discuss the utility of CD229, CD319 and CD54 as potential complements or alternatives to the mostly used markers in identification of PC. The SLAM family member CD229 (Ly9) was reported to be expressed by T-, NK-, B- cells, macrophages and dendritic cells,

in addition to primary MM cells [14-15, 18]. It could be strongly over-expressed and phosphorylated in MM cell lines; as reported by Atanackovic et al. [14] anti-CD229 monoclonal antibody produce complement- and cell-mediated lysis of myeloma cells, suggesting that CD229 may be of potential utility for the treatment of MM [14]. CD319 (CS1), another member of the SLAM family, is also expressed by normal NK- and B-cells, by activated CD4 and CD8 T- cells, and by DC [15, 18]. CD319 (CS1) is reported to be expressed at high levels on myeloma cells independently of the molecular background [15], which has lead Frigyesi et al.[24] to propose CD319 to be a more robust marker than CD138 for PC isolation [24]. At present clinical trials are ongoing which use the anti-CS1 mAb elotuzumab [15]. CD54 (ICAM-1) is a well chasracterized adhesion molecule which is broadly expressed on leukocytes and endothelial cells [19]. As regards PC, CD54 has been shown to be expressed on PC from healthy volunteers, MM cell lines, and primary MM PC [16]. Veitonmäki et al. [17] reported that an antibody specifically targeting ICAM-1, BI-505, exhibited a pronounced antimyeloma activity , and this antitumour function was macrophage-dependent [17]. In our experimental setting, expression of CD229 was persistently positive in both BM and PB PC. We observed that full positivity is preserved in BM samples stained with APC conjugated antibodies, while in 1.1% of samples analyzed with the use of PerCP-Cy5.5 conjugate in combination with fixation/permeabilization technique some PC showed no staining. It's reasonably to speculate that in these samples, the use of a less sensitive fluorochrome compared with APC and fixation/permeabilization protocol might have played a role. In BM and PB samples, CD229 was also expressed by B cells, T cells subsets and pDC, while it was absent on monocytes, eosinophils and all stages of maturing neutrophils. This is in line with previous observations which also showed expression of CD229 on tissue macrophages [14-15, 18]. In turn, CD319 and CD54 were uniformly positive only on BM PC. Although none of the three markers showed clear expression differences between normal and clonal PC populations, they show value in identifying PC. When used together with CD38 in the initial gate, the three markers allow an efficient identification of PC, which can be precisely defined using further backgating in other classic recommended representations, like CD38 vs SSC, CD38 vs CD45 and FSC vs SSC [20]; so the combinations CD229 vs CD38, CD319 vs CD38 and CD54 vs CD38 may be considered as potent alternatives to CD38 vs CD138, especially in cases particularly prone to loss or downregulation of CD138, and patients undergoing to anti-CD138 therapy. Moreover we explored the potential values of CD229, CD54 and CD319 as alternatives to CD38 in initial PC MFC separation from all other BM and PB cell types. With CD54/CD319 vs SSC or CD138 gate, the major problem is detection of all aPC, since on aPC levels of expression are lower compared to nPC, despite the fact that no significant difference has been observed, and partially overlapping with other non-PC populations. On the contrary, although initial PC identification gate is contaminated with lymphocytes and pDC, exclusion of non-PC events was more efficient using

CD229 vs SSC and CD138 when compared with CD54/CD319 vs SSC and CD54/CD319 vs CD138.

We propose that CD229 should be considered in further studies on patients undergoing to anti-CD38 therapies in order to assess its possible use in clinical settings.

In conclusion, we have provided the basis for the investigation of these new markers in the MFC identification of PC, especially in those cases in which CD138 or CD38 expression are lost or compromised. We have also demonstrated that CD229 could be considered as a surface antigen able to bypass the need of CD38 in PC detection and needs to be tested in clinical practice, so more efforts should be performed in this sense in future.

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Boyero R, Galende J, Hernández J, Palomera L, Carrera D, Martínez R, de la Rubia J, Martín A, González Y, Bladé J, Lahuerta JJ, Orfao A, San-Miguel JF; GEM (Grupo Español de MM)/PETHEMA (Programa para el Estudio de la Terapéutica en Hemopatías Malignas) Cooperative Study Groups. The persistence of immunophenotypically normal residual bone marrow plasma cells at diagnosis identifies a good prognostic subgroup of symptomatic multiple myeloma patients. Blood. 2009 Nov 12;114(20):4369-72. doi: 10.1182/blood-2009-05-221689. Epub 2009 Sep 15. PubMed PMID: 19755674.

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Table 1. Different percentage of PC positive for identification markers

	HC PB[1]	HC BM[1]	PCD BM[1]		NI-NHL BM[2]	PCD BM[2]		EMP[3]	BM[3]	
MARKER	nPC	nPC	nPC	aPC	nPC	nPC	aPC	aPC	nPC	aPC
CD38	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)
CD138	29% (24%-69%)	98% (97%-100%)	97% (50%-100%)	99% (79%-100%)	99% (97%-100%)	100% (91%-100%)	100% (96%-100%)	100% (100%-100%)	95% (47%-100%)	100% (76%-100%)
CD54	85% (67%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)						
CD319	100% (86%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)			
CD229	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)	100% (100%-100%)				100% (100%-100%)	100% (84%-100%)	100% (73%-100%)

[1]5HC BM, 1SMM, 2MGUS, 2MM, 5HC PB samples; [2]1 MM, 3 MRD, 1 MGUS, 2 NI-NHL samples; [3]3 MM, 1 MGUS, 1 PCL, 3 EMP, 1 SBP, 30 MRD. HC, healthy control; PB, peripheral blood; HC, BM, bone marrow; NI-NHL, not infiltrated BM from non-Hodgkin lymphoma patients; PCD, plasma cell dyscrasia; EMP, extraosseous (extramedullary) plasmocytoma; nPC, normal plasma cells; aPC aberrant plasma cells.

Supplementary Table I. Clones and manufacturer of antibodies used in this report.

MARKER	FLUOROCHROME	CLONE	MANUFACTURER
CD3	PerCP-Cy5.5	SK7	BD
CD4	PacB	RPA-T4	BD
CD8	FITC	RPA-T8	BD
CD10	APC H7	HI10a	BD
CD11b	FITC	Bear1	BC
CD13	PE	L138	BD
CD14	APC H7	M□P9	BD
CD16	PE-Cy7	3G8	BD
CD19	APC H7	SJ25C1	BC
	PE-Cy7	J3-119	Immunostep
CD27	BV421	M-T271	BD
CD20	PacB	2H7	BioLegend
CD34	PerCP-Cy5.5	8G12	BD
	APC-A750	581	BC
CD38	FITC	LD38	Cytognos
	PacB	HIT2	ExBio
	V450	HB7	BD
	APC H7	HB7	BD
CD45	PacB	T29/33	Dako
	APC	ML2	Cytognos
	BV510	HI30	BD
CD54	PE	3E2	BD
CD56	PE	C5.9	Cytognos
	APC-Cy7	HCD56	BioLegend
	PE-Cy7	B159	BD
CD64	FITC	32.2	BC
CD117	APC-A750	104D2D1	BC
CD123	BV421	9F5	BD
CD138	PerCP-Cy5.5	MI15	BD
	PacO	B-A38	ExBio
	V450	MI15	BD
CD229	APC	HLy9.25	BioLegend
	PerCP-Cy5.5	HLy9.25	eBioscience
CD300e	PE	UP-H2	Immunostep
CD319	FITC	162	ABD SEROTEC
	PE	162	eBioscience
anti-κ	APC	Polyclonal	Dako
anti-λ	APC H7	1-155-2	BD
HLA DR	PerCP-Cy5.5	L243	BD
	PacB	L243	BioLegend
IgM	FITC	Polyclonal	Dako
IgD	PerCP-Cy5.5	IA6-2	BioLegend

TCRγδ	PE-Cy7		11F2	BD				
Panel	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	PacB	PacO
1[a]	CD319	CD54	CD138	CD19	CD229	CD56*	CD38[#]	CD45[§]
2[b]		CD319		CD56	CD45	CD19	CD38	CD138
3[c]	CD38	CD56	CD229	CD19	Cytκ	Cytλ	CD45	CD138
4[d]	CD8	CD56	CD3	CD19-TCRγδ	CD229	CD14	CD4-CD20	CD45[§]
5[d]	sIgM	CD56	sIgD	CD19	CD229	CD38	CD27[$]	CD45[§]
6[e]	CD64	IREM2	HLA DR	CD16	CD229	CD14-CD34[&]	CD123[$]	CD45[§]
7[f]	CD11b	CD13	CD34	CD16	CD229	CD117[&]	HLA DR	CD45[§]
8[f]	CD38	CD56	CD34	CD19	CD229	CD10	CD138[#]	CD45[§]

PerCP-Cy5.5, Peridinin-chlorophyll protein-Cyanine5.5; PacB, Pacific Blue; FITC, Fluorescein isothiocyanate; APC, Allophycocyanin; H7, Hilite7; PE, R-phycoerythrin; Cy7, Cyanine7; APC-A750, APC-Alexa Fluor 750; BV421, Brilliant Violet 421; BV510, Brilliant Violet 510; PacO, Pacific Orange. *APC-Cy7 conjugate; &APC-Alexa Fluor 750 conjugate; #V450 conjugate; $BV421 conjugate; §BV510, Brilliant Violet 510 conjugate.

BD, BD BIoscience (San Jose, CA, Usa); BC, Beckman Coulter (Brea, CA, USA); Immunostep (Salamanca, Spain); Biolegend (London, UK); Cytognos SL, (Salamanca, Spain); Dako (Glostrup, Denmark); ExBio (Vestec, Czech Republic); eBioscience (San Diego, CA, USA); ABD Serotec (Kidlington, UK).

[a]5HC BM, 1SMM, 2MGUS, 2MM, 5HC PB samples; [b]1 MM, 3 MRD, 1 MGUS, 2 NI-NHL samples; [c]3 MM, 1 MGUS, 1 PCL, 3 EMP, 1 SBP, 30 MRD; [d]5HC PB; [e]5 HC PB and 5 HC BM; [f]5 HC BM.

HC, healthy controls; BM, bone marrow; PB, peripheral blood; N°, number of samples; SMM, smoldering myeloma; MGUS, monoclonal gammopathy of uncertain significance; MM, multiple myeloma; NI-NHL, not infiltrated BM from non-Hodgkin lymphoma patients; MRD, minimal residual disease evaluation cases; EMP, extraosseous (extramedullary) plasmocytoma; SBP, solitary bone plasmocytoma; PCL, plasma cell leukemia. Cyt, cytoplasmic; κ, kappa light chain; λ, lambda light chain.

Figures

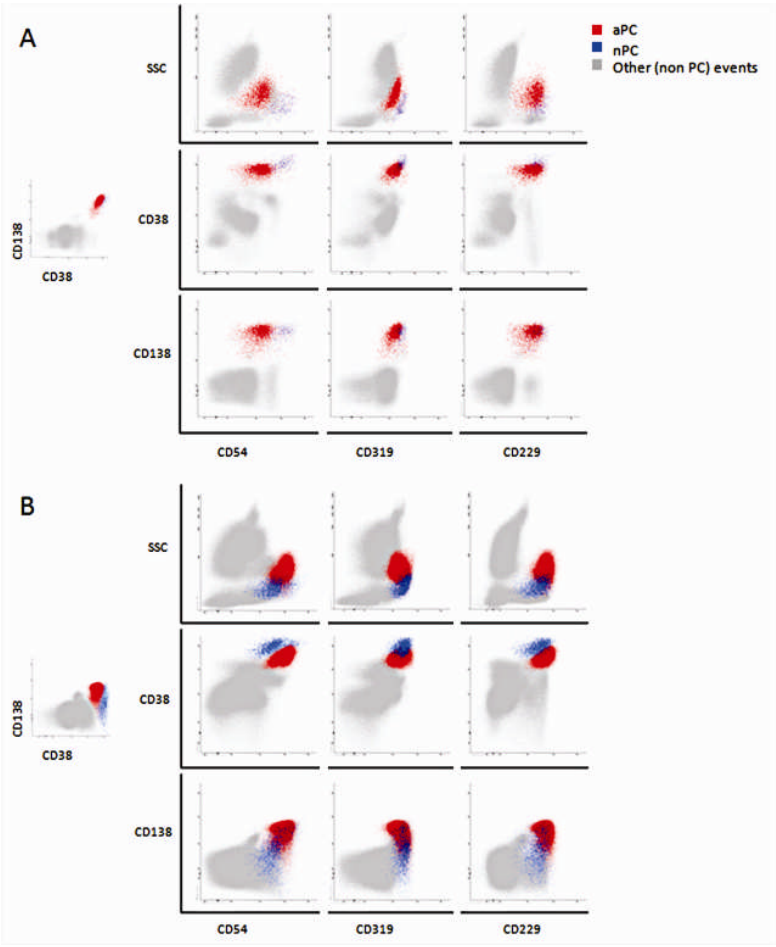

Figure 1. Illustrating examples of the expression of CD54, CD319 and CD229 on BM populations of PC from 2 multiple myeloma patient samples stained with the first panel. The three markers evaluated are plotted against SSC, CD38 and CD138, respectively. Panel A and B correspond each to a different patient. Please note that a lower intensity of aPC occurs for CD54 in panel A and for CD319 in both panel A and B. In turn, PC from panel A show higher CD138 expression than in the case displayed in panel B. As may be seen, CD54 and CD319 showed a poor discrimination of PC from the other BM cells vs CD229, mostly because of the stronger staining index on PC vs the myeloid BM cell compartment. PC, plasma cells; SSC, side scatter; nPC, normal plasma cells; aPC, aberrant plasma cells.

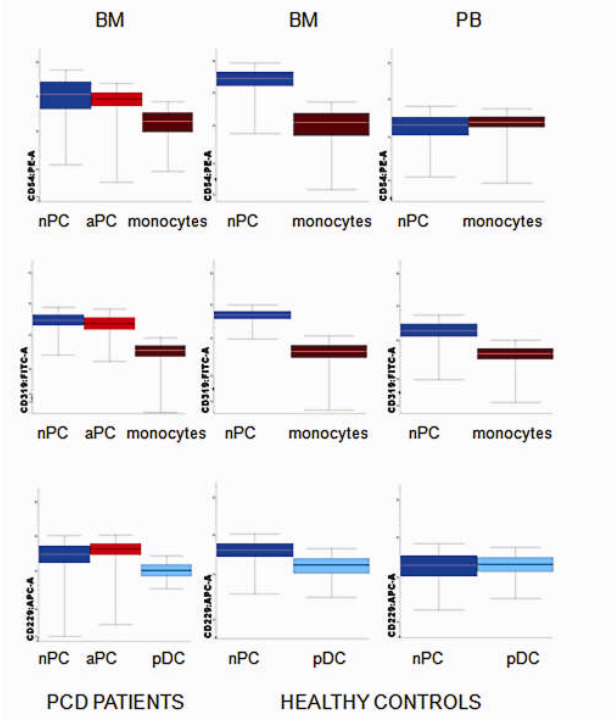

Figure 2. Levels of expression (Fluorescence intensity – arbitrary units) of CD54 (A), CD319 (B) and CD229 (C) in PB and BM plasma cells from healthy controls and PCD patients compared with monocytes and plasmacytoid dendritic cells (pDC). This graphical representation was obtained performing a merge operation of 5 PCD BM, 5 HC BM and 5 HC PB, respectively. All samples were stained with panel 1. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. nPC, normal plasma cells; aPC, aberrant plasma cells.

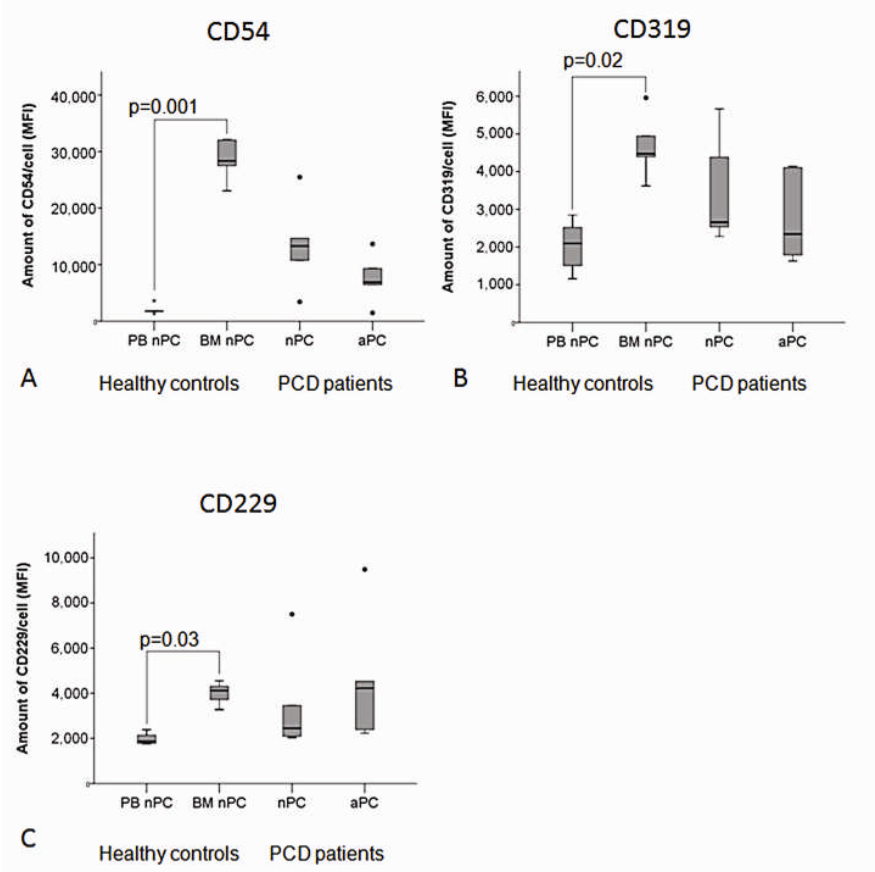

Figure 3. Levels of expression of CD54 (A), CD319 (B) and CD229 (C) in PB and BM plasma cells from healthy controls and PCD patients (antibody panel 1). Results are expressed as median fluorescence intensity (arbitrary units scaled from 0 to 262 144). Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. Black dots and stars indicate the outliers. PC, plasma cells; PB, peripheral blood; BM, bone marrow; nPC, normal plasma cells; aPC, aberrant plasma cells; PCD, plasma cell disorders.

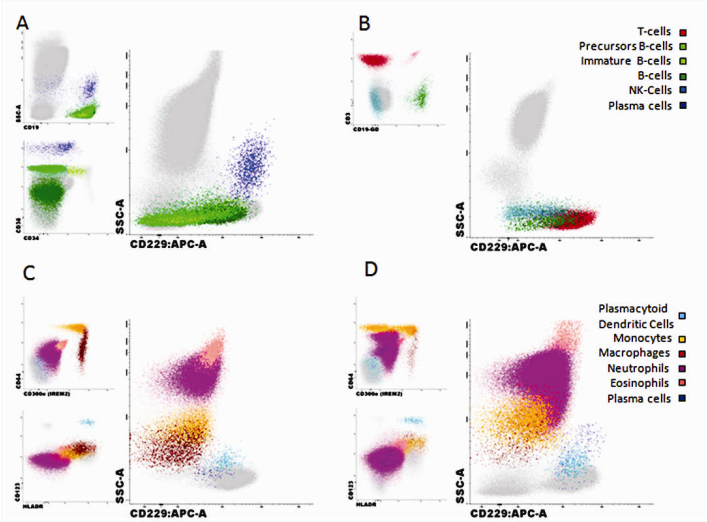

Figure 4. Illustrating bivariate dot plots showing CD229 expression on non PC PB and BM cell populations vs PC. Panels A, BM B-cells are displayed (green dots). Panel B shows expression of CD229 on PB B- (green dots), T- (red dots) and NK-cells (cyan dots) vs PC (blue dots). In turn, expression of CD229 major myeloid populations present in PB and BM - on monocytes (yellow dots) and CD16+ monocytes (brown dots), neutrophils (fuchsia dots), plasmacytoid dendritic cells (pale blue dots) and eosinophils (pink dots)- vs PC (blue dots) is depicted in panels C and D, respectively. Of note, CD229 expression among the PB and BM myeloid cell compartment was restricted to plasmacytoid dendritic cells. No positivity was observed on macrophages, monocytes, neutrophils and eosinophils.

5. Bone marrow B cell subsets in MGUS and multiple myeloma: focus on reorganization of a forgotten immune branch

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TITLE

Bone marrow B cell subsets in MGUS and multiple myeloma: focus on reorganization of a forgotten immune branch

RUNNING TITLE

Bone marrow B cells in MGUS and myeloma

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ABSTRACT

Multiple myeloma (MM) is an incurable disease principally of older patients, caused by the proliferation of clonal plasma cells (cPCs) in bone marrow (BM). Expansion of cPCs causes not only several systemic disorders, but also numerical and functional defects in many immune subsets. Although T lymphocytes and dendritic cells are the most studied lineages, also an impairment of B cell compartment is involved in onset and progression of the disease. We studied the distribution of naïve/transitional (IgD+CD27−), memory unswitched (IgD+CD27+), memory switched (IgD−CD27+) and double negative (DN) (IgD−CD27−) B lymphocytes in BM of control subjects and different categories of responding and relapsing patients. We observed an increased percentage of IgD−CD27+ in healthy controls compared with responding patients. The IgD+CD27− subpopulation was significantly larger in stringent complete responders than in other treated patients; the situation was inverted for DN B cells, with treated patients exhibiting an expanded compartment compared to deep responders. None of the studied B cell subsets exhibited clonal restriction. We have provided a feasible start point to explore the importance of B cells in the immunosuppressive MM BM microenvironment. Moreover, we propose a possible role of naïve/transitional and DN B cells as predictive markers in treated patients.

KEYWORDS:

Multiple Myeloma

MGUS

B cells

Introduction

Multiple myeloma (MM) is a disease principally of older patients, with a median age at diagnosis of 65–70 years. It is caused by proliferation of clonal plasma cells (PCs) in the bone marrow, associated with the presence of monoclonal immunoglobulin (Ig) in the serum or urine and signs of multi-organ impairment that can include anemia, lytic bone lesions, immunodeficiency, and reduced renal function [1-2]. MM is preceded by an asymptomatic premalignant stage, called monoclonal gammopathy of undetermined significance (MGUS), which progresses to myeloma at a rate of 1% per year [1]. Progression to MM is characterized by a series of complex genetic events in MM clones and changes in the bone marrow microenvironment, including (and most notably) suppression of the immune response [1-2]. Immunosenescence is defined as the insurgence of deterioration in the normal functions of the immune system in parallel with physiologic ageing [3]. It progresses with changes in adaptive immune functions mediated by T and B cells in the elderly, directly influenced by environmental and genetic factors, and by the antigenic pressure to which individuals are exposed during their entire life [3-4]. This phenomenon causes an impaired ability to respond to vaccines and new infectious agents, and an increased susceptibility to infectious diseases, autoimmunity and cancer [3-5]. The immune system of the elderly has been extensively studied, for the most part involving the T cell branch, however B cell compartment is also defective in the elderly: humoral immune response differs both in the quality (in terms of production of high affinity responses) and levels of the produced antibodies, and the number of circulating B cells is reduced in the aged [3-4]. The reduction in percentage of naïve B lymphocytes (IgD+CD27−) and the increase in percentage of a "double negative" (DN, IgD−CD27−) memory B cell population have been demonstrated in peripheral blood of the elderly [6]. This DN population seems to be an "exhausted" memory population [4, 6-8]. As reported by Bulati et al. [7], it has been hypothesized that DN B cells are involved in the inflammatory environment related to aging and that they might be a by-product of systemic inflammation or directly involved in the immune response [7]. In multiple myeloma field, most of the studies have focused on T regulatory and dendritic cells (DCs), in order to explain the immunosuppressive BM microenvironment that allows the expansion of the neoplastic clone [9-10]. To the best of our knowledge, nobody has already explored the distribution of B cell subsets and presence of DN B cells in bone marrow of MM subjects in different stages of the disease. In this paper we report the distribution of B cell subsets in BM of healthy subjects, and MGUS, newly diagnosed, responding and relapsing MM patients. Moreover we also evaluate the possible presence of clonal restriction in these lymphocytic subpopulations.

Materials and Methods

Patients and BM samples

Control specimens consisted of 9 BM samples from patients who were suspected to have a haematological disease and revealed to be non onco-hematological subjects (group Control). These patients have no history of MM, MGUS or lymphoid/myeloid neoplasm. BM samples of 44 patients with MM and 10 patients with MGUS submitted to our laboratory for routine analysis were evaluated by multiparameter flow cytometry (MFC). For every patient clinical chemical and immunological profiles, as well as reference intervals were provided by the U.O. Patologia Clinica – Laboratorio Analisi Cliniche of ARNAS Civico, Palermo (Italy). Disease stage was defined according to Durie-Salmon and ISS staging criteria [11-12]. Response to therapy was defined according to Bird et al. [13]. Of MM samples, 12 were obtained at presentation (group New), 8 from patients with progressive disease (group Progressive), 14 from patients in partial remission (4 Very Good Partial Response, 6 Partial Response and 4 Stable Disease - group Therapy) and 10 from patients who achieved stringent CR (group Complete). MGUS patients were considered as a separate group (MGUS group). Clinical data and history for MGUS and MM cases were provided by U.O. Oncoematologia of ARNAS Civico, Palermo (Italy). Informed consent procedures and forms were proposed to and approved by the ARNAS Civico Medical Ethics Committee. Written informed consent was given by all subjects in line with the Declaration of Helsinki Protocol. BM samples were collected in EDTA tubes and processed within one hour since collection.

Multiparameter Flow Cytometry

Details about the instrument and antibodies used in this study are summarized in Supplementary Table 1 and Supplementary Table 2 respectively. Specimens were fragmented with a sterile syringe and filtered using a 80μm filter; nucleated cells were enumerated using UniCel® DxH™ 800 Coulter® Cellular Analysis System (Beckman Coulter, Miami, FL, USA) and brought to a final concentration of 10^6cells/100μl with PBS w/o calcium and magnesium (EuroClone, Milan, Italy). Combinations of antibodies used to stain surface and intracellular markers were: Tube 1, $Cyt\kappa_{FITC}$/$Cyt\lambda_{PE}$/$CD38_{PC5.5}$/$CD56_{PC7}$/$CD138_{APC}$/$CD27_{APC\text{-}AlexaFluor\ 750}$/$CD19_{PB}$/$CD45_{KO}$; Tube 2, $CD27_{FITC}^{\#}$/$IgD_{PE}^{\#}$/$CD38_{PC5.5}$/$CD10_{PC7}^{\#}$/$CD138_{APC}$/$CD34_{APC\text{-}AlexaFluor\ 750}$/$CD19_{PB}$/$CD45_{KO}$; Tube 3 $Cyt\kappa_{FITC}$/$Cyt\lambda_{PE}$/$CD10_{ECD}$/$CD38_{PC5.5}$/$IgD_{PC7}^{\#}$/$CD138_{APC}$/$CD34_{APC\text{-}AlexaFluor\ 700}$/$CD27_{APC\text{-}AlexaFluor\ 750}$/$CD19_{PB}$/$CD45_{KO}$ (Cyt, Cytoplasmic; FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas Red; PC5.5, Phycoerythrin-Cyanin 5.5; PC7, Phycoerythrin-Cyanin 7; APC, Allophycocyanin; PB, Pacific Blue; KO, Krome Orange). All antibodies were purchased from Beckman Coulter (Miami, FL, USA), except those with # which were purchased from BD Biosciences (San Jose, CA, USA). For staining of surface markers, 100μl of each sample were

incubated with the appropriate combinations of antibodies for 15 minutes in the dark. Erythrocytes were lysed adding 1 ml of VersaLyse™ Lysing Solution (Beckman Coulter, Miami, FL, USA) and incubating tubes for 20 minutes in the dark. For intracellular staining of kappa and lambda light chains, 50µl of sample were washed 5 times with 2 ml of PBS w/o calcium and magnesium (EuroClone, Milan, Italy), and processed with PerFix-nc (Beckman Coulter, Miami, FL, USA) following instructions. Samples were all acquired with Navios™ Flow Cytometry System (Beckman Coulter, Miami, FL, USA), data were collected with Navios v1.0 Software (Beckman Coulter, Miami, FL, USA) and then analyzed with Kaluza® Flow Cytometry Analysis Software v1.3 (Beckman Coulter, Miami, FL, USA). Daily testing of instrument was performed as indicated: standardization of light scatter, fluorescence intensity and optimal hydrodynamic focusing instrument settings were verified using Flow-Set Pro Fluorospheres (Beckman Coulter, Miami, FL, USA); compensation matrix for each combination of antibodies was tested with CYTO-COMP™ Cell Kit (Beckman Coulter, Miami, FL, USA); optical alignment and fluidics were checked using Flow-Check Pro Fluorospheres (Beckman Coulter, Miami, FL, USA). In order to identify PCs, a combination of CD38, CD138 and CD45 together with side scatter properties was used; the first gate was set on CD38vsCD138 as suggested [14]. Distinction between normal/reactive (nPC) and clonal plasma (cPC) cell compartments was performed basing on their most frequent aberrant phenotypes (as regards CD38, CD19, CD27, CD56 and CD45); results were confirmed by the presence of clonal restriction in population showing the abnormal phenotype, and the absence of restriction in normal PCs [14-15]. The κ:λ ratio was defined as abnormal if < 0.5 or > 3 [16]. In tube 2 and 3, B cells were selected setting the first gate on CD19+ cells in CD19vsSSC plot; then precursors and immature B cells were excluded from the analysis in order to restrict the study to naïve/transitional B cells, and switched/unswitched and DN memory B cells (in this paper called on the whole "total B cells"), according to the recognized phenotypes: **IgD+CD27-**CD10± **transitional**, **IgD+CD27-**CD10-CD38-/+dim **naïve**, **CD27+ switched/unswitched memory** and **CD27-IgD- double negative B cells** [17]. All these subsets together are indicated in this paper as total B cells. A minimum of 200 events in the plasma cell gate and 20000 events in the mature lymphocyte gate in CD45vsSSC plot were collected for each tube; in order to reach this result, a total of 200,000-2,000,000 events were acquired. For each marker, an internal negative population present within the sample was used to define gates and sample fluorescence background [18-19]. Data were measured as percentage of cells presenting the antigen (percentage of positive cells).

Statistical analysis

Continuous non normal data are expressed as median values (range); normal variables are indicated as mean±SD. Baseline differences between groups were assessed by the chi-square test or Fisher's

exact test, as needed for categorical variables (with Bonferroni correction for multiple comparisons). The univariate analysis of variance (ANOVA) was performed for parametric variables, and post hoc analysis with the Tukey's test was used to determine pairwise differences. The Kruskal-Wallis statistic test with pairwise comparisons was performed for nonparametric analysis. Data were analyzed by IBM SPSS Software 22 version (IBM Corp., Armonk, NY, USA). All p-values were two-sided and $p<0.05$ was considered statistically significant.

Results

Characteristics of patients

As indicated in Table 1, no significant difference were found about sex distribution, comorbidities and ISS staging at diagnosis, but Durie-Salmon stage IIIA was met more frequently in Complete than in New patients. Autologous stem cell transplantation was performed in 6 Complete, 6 Therapy and 2 Progressive patients at least 12 months before the time of this study (not significant). Data inherent administered therapeutic regimens were recorded, but no statistically significant differences were detected. Bortezomib+Dexamethasone based treatment was administered to 5 Therapy and 3 Progressive patients. Thalidomide as monotherapy or in combination with bortezomib was given to 1 Complete and 3 Therapy patients respectively. Lenalidomide or lenalidomide containing regimens were the treatment of choice in 4 Complete and 1 Progressive subjects, while Pomalidomide was administered to 1 Progressive patient. In addition 5 Complete, 6 Therapy and 2 Progressive patients underwent suspension of therapy before for at least 15 days before this study was performed. Median percentage of total PCs was significantly higher in New [8.64% (0.26-60.50)] and Progressive [6.47% (0.75-30.29)] compared with Control [0.15% (0.01-1.71), vs New $p<0.0005$, vs Progressive $p=0.001$], Complete [0.38% (0.03-2.49), vs New $p=0.006$, vs Progressive $p=0.013$] and Therapy [0.48% (0.12-14.40), vs New $p=0.012$ and vs Progressive $p=0.029$], while no difference was observed with MGUS [1.39% (0.36-2.40)]. All PCs in Control and Complete were polyclonal, while nPCs were 19.12% (5.24-82.52) in MGUS, 29.24% (0.14-96.12) in Therapy, 1.18% (0.05-66.12) in New (vs Therapy $p=0.016$), and 1.60% (0.25-12.88) in Progressive. As regards cPCs, New showed the highest median percentage [98.83% (33.88-99.95)] compared with Therapy [70.76% (3.88-99.86), $p=0.016$], but no difference emerged with MGUS [80.88% (17.48-94.76)] and Progressive [98.40% (87.12-99.75)].

Distribution of B cells among groups and relationship with the neoplastic clone.

Median percentage of total B cells, naïve/transitional (IgD+CD27−), memory unswitched (IgD+CD27+), memory switched (IgD−CD27+) and DN (IgD−CD27−) B lymphocytes and their

relative ranges are reported in Figure 1 (A-E). Progressive showed the most modest percentage of total B cells compared with Control and New. Control patients showed the highest percentage of memory unswitched cells compared with Complete and Therapy. Naïve/Transitional subpopulation was significantly larger in Complete than in Therapy patients; the situation was inverted for DN B cells, with Therapy exhibiting an expanded compartment compared to Complete. Moreover DN B cells were more numerous in Therapy than in Control subjects. No differences were observed for memory switched B cells among groups. In order to explore at a preliminary level a possible relationship with the neoplastic clone, a total of 10 patients (4 New, 2 MGUS, 3 Therapy and 1 Progressive) underwent clonal restriction assessment in B cell subsets. No patient exhibited signs of clonal restriction in all studied subpopulations (Figure 1F).

Discussion

In MM patients, the expansion of cPCs in the BM, and consequential alteration of the homeostatic equilibrium of both staminal hematopoietic/stromal and immune compartments, causes not only end organ damage (including increased calcium levels, defective renal function, anemia, and lytic bone lesions), but also defects in immune system; many of these alterations are reported to be associated with poor disease outcome [1, 9, 10]. Tumour-rejection immune responses of the earlier phases of the disease are gradually evaded, and ultimately are defeated in their battle to stop and eradicate the proliferating plasma cell clones [10]. Multiple myeloma is associated with cellular and humoral immune deficiencies. Patients frequently exhibit poor response to vaccination against influenza, *Streptococcus pneumoniae* and *Haemophilus influenzae* type B [10]. Many (soluble or not) mediators of the bone marrow immunological microenvironment (including TGF-β, IL-10, VEGF, MUC-1 and PGE2) and direct contact are involved in supporting the malignant microenvironment and in suppression of the host anti-myeloma immune response [1, 10]. A number of efforts have been made to characterize disfunctions in T cell compartment, and the role of DCs and myeloid derived suppressor cells (MDSCs) [9-10, 20-21], but B cell subsets were less considered in describing reciprocal interactions between the immune system and myeloma cells. We found that healthy subjects showed a higher percentage of IgD+CD27+ memory cells compared to the two responding groups (Therapy and Complete), but no difference with pre-malignant group MGUS, and with newly diagnosed and relapsing subjects. IgD+IgM−CD27+ memory cells appear to play an important role in respiratory mucosal defense via, while CD27+IgM+IgD+ memory cells are involved in the response to encapsulated bacteria [22]. With this in mind, we find our result is an intriguing piece of data in order to discover alternative explanations to increased susceptibility to infections in MM patients [23-24], especially in the look for mechanisms which could be specific of responding patients. In fact, it has already been

documented that anti-myeloma therapies exhibit a profound impact on immune system and emergence of infections [24], but a well dissected relationship among effect of therapy-bone marrow B cell subset distribution-depth of response is still missing. Naïve/Transitional cells were expanded in Complete vs Therapy patients, showing a possible reorganization of B cell compartment in patients who do not show signs of BM neoplastic clone. It has been demonstrated in the elderly that naïve/transitional B cells are highly activated to produce both IL10 and TNF-α under physiological (anti-CD40 and IL4) stimulation [8], so it will be interesting to evaluate the impact of this subpopulation in modulating inflammation and its resolution in MM patients. As regards DN B cells, observed results revealed an increase of this subset in Therapy patients compared to Complete. This is not surprising, since DN B cells are described as exhausted B lymphocyte population filling the immunological space especially in elderly subjects. DN B cells have also been reported to be expanded in subjects affected by SLE and challenged with RSV [4], and presented a very low ability to be activated by different stimuli, including physiological ones [4, 6-8]. Moreover these cells exhibit a tissue trafficking phenotype and can be stimulated to secrete granzyme B, which plays a critical role in cancer immunosurvellance [7]. Differences in percentage of DN B cells may reflect the ability of various categories of patients to reach stringent complete response in the MM scenario. Considering our results, monitoring fluctuations in percentage of Naïve/Transitional and DN B cells may reveal a useful tool in predicting the type of response, although larger cohort studies are necessary to identify a cut-off value in order to distinguish stringent complete from other responding patients. Some works have reported conflicting results about clonotypic CD19+CD34+ or CD19+CD34- B cell, which are considered as a non plasmacellular proliferating MM compartment [25-28]. So we decided to investigate at a preliminary level if B cell subsets showed clonal restriction. We observed that none of the studied patients showed signs of expansion of a clonal B population. Although this result must be confirmed by deeper molecular studies, it is really interesting since it would exclude the presence of a relation between B cells and neoplastic clone, opening the space to new speculations about the interplay involving MM PCs and pre and post switch B lymphocytes. In conclusion we have provided an elegant start point to explore the role of B cells in the immunosuppressive MM BM microenvironment. Moreover, we propose a possible role of Naïve/Transitional and DN B cells as predictive markers in treated patients.

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Caterina Giambanco, Francesco Gervasi, Matteo Bulati, Silvio Buffa, Giuseppina Colonna Romano and Calogero Caruso revised the paper; all authors approved the paper. Authors would like to thank James Hinchcliffe for reviewing language and syntax of the manuscript, and Filippo Pojero for elaborating artworks.

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Tables

Table 1. Characteristics of subjects included in this study

		Control	MGUS	Complete	Therapy	New	Progressive	p Value	
N° of cases		9	10	10	14	12	8		
Age (years)		62± 5.36	70.8± 7.3	61.1± 8.85	61.21± 9.01	63.58± 12.8	75.13± 10.06	Cp vs P T vs P	0.029 0.017
Sex	Male	6	7	3	11	6	6	NS	
	Female	3	3	7	3	6	2		
Subtype	IgA λ		1	1	1	3	2		
	IgA κ				4		3		
	IgG λ		3	2	4	6			
	IgG κ		6	3	3	2	3		
	λ			3	2	1			
	κ			1					
D-S Stage	IA			2		4	4	NS	
	IIA			1	5	5	2	NS	
	IIB				3	2	2	NS	
	IIIA			6	2			Cp vs N	0.018
	IIIB			1	4	1		NS	
ISS Stage	I			3	3	3		NS	
	II			7	4	5	5	NS	
	III				7	4	3	NS	
Comorbidities	CRI		2		1	1	2	NS	
	Diabetes		2	1	2	2	4	NS	
	Hypothyroidism			1	2			NS	
	Obesity		1	2	1			NS	
	Cardiopathy		1			1	3	NS	
	HCV		1		1			NS	
	HBV				2	2		NS	
	Thalassemia Trait				1			NS	
	Pregress Cancer		2		2	1		NS	

D-S Stage, Durie Salmon stage; CRI, chronic renal insufficiency; HCV, HCV related chronic hepatitis; HBV, HBV related chronic hepatitis; Pregress Cancer, patients who reported non hematological types of cancer in the last 10 years; Ct, Control; M, MGUS; Cp, Complete; T, Therapy; N, New; P, Progressive. NS, not significant.

Figure

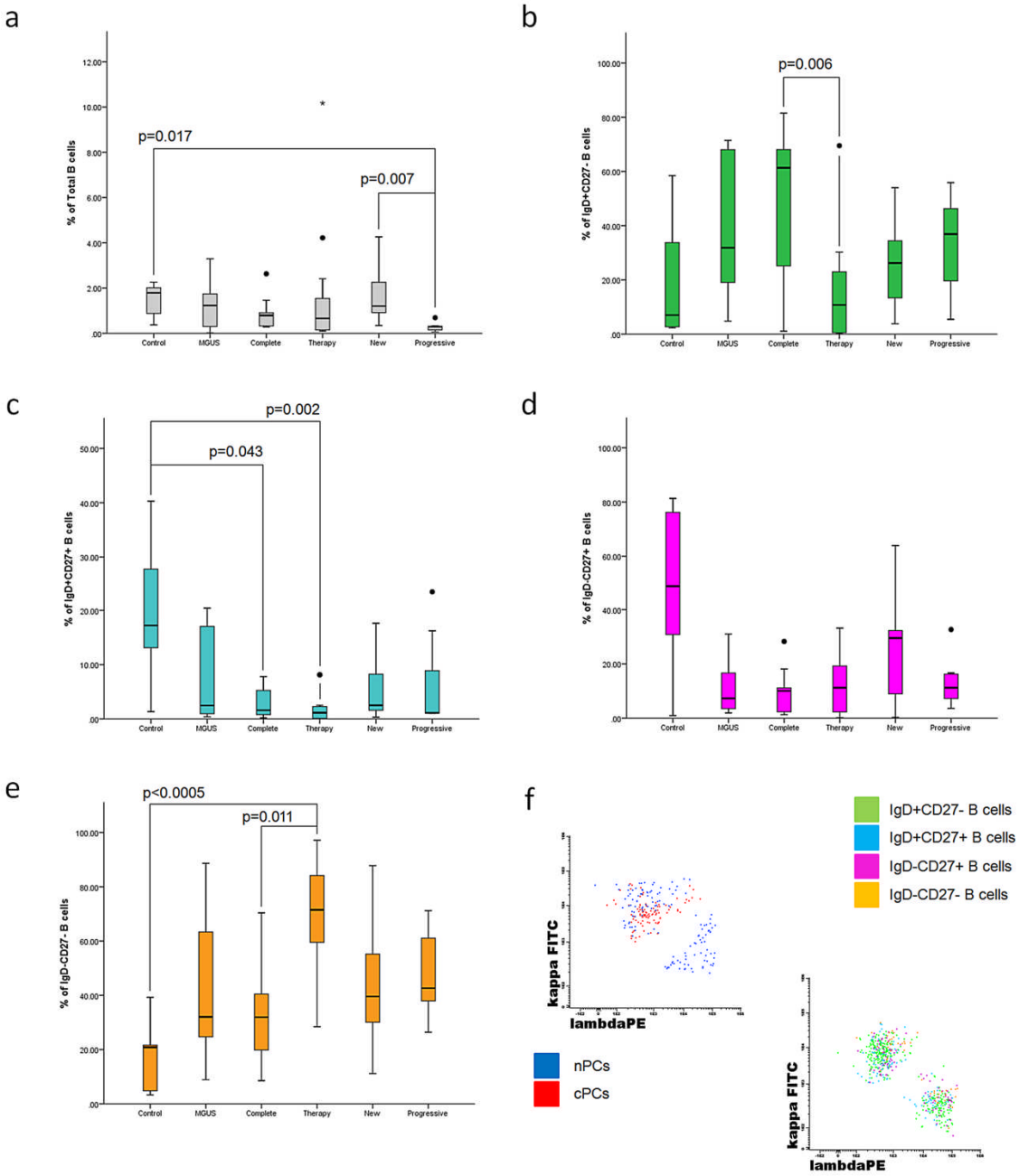

Fig. 1 Results of statistical analysis performed on total B cells and the various subpopulations. Box plots and p values are reported. Moreover, an exemplificative dot plot displaying clonal restriction analysis is depicted. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. To follow, median percentage [range] of total B cells and different subsets. a) Total B cells: Control 1.79% [0.37-2.26], MGUS 1.23% [0.01-3.30], Complete 0.79% [0.29-2.63], Therapy 0.66% [0.10-10.17], New 1.20% [0.34-4.26], Progressive 0.29% [0.06-0.69]. b) IgD+CD27- (naïve/transitional) B cells: Control 7.01% [2.31-58.47], MGUS 31.88% [4.78-71.46], Complete 61.33% [1.10-81.53], Therapy 10.79% [0.12-69.51], New 26.25% [3.85-54.01], Progressive 36.89% [5.45-55.90].c) IgD+CD27+ (memory unswitched) B cells: Control 17.22% [1.34-40.29], MGUS 2.48% [0.39-20.41], Complete 1.61% [0.19-

7.79], Therapy 1.14% [0.08-8.16], New 2.51% [0.31-17.63], Progressive 1.11% [0.99-23.42]. d) IgD-CD27+ (memory switched) B cells: Control 48.63% [0.94-81.31], MGUS 7.31% [1.92-31.01], Complete 10.08% [1.30-28.28], Therapy 11.23% [0.21-33.18], New 29.52% [0.31-63.94], Progressive 11.23% [3.60-32.67]. e) IgD-CD27- (Double Negative) B cells: Control 20.87% [3.27-39.25], MGUS 32.02% [8.90-88.64], Complete 31.95% [8.53-70.41], Therapy 71.49% [28.42-97.18], New 39.57% [11.17-87.77], Progressive 42.64% [26.39-71.21]. f) Exemplificative bivariate dot plots of a MGUS case showing clonal restriction in cPCs and absence of restriction in all B cell subsets (bivariate dot plots were elaborated with Infinicyt flow cytometry software version 1.3, Cytognos SL, Salamanca, Spain)

Supplementary Table 1. Characteristics of the instrument.

Model (manufacturer)	Lasers	Detector Filters
Navios (Beckman Coulter) 10 colors, 3 lasers (5+3+2 configuration)	Blue Solid State Diode: 488nm, 22mW laser output	Forward Scatter: 488/10 Blue Laser: 525/40, 575/30, 620/30, 675/20, 695/30, 755LP
	Red Solid State Diode: 638nm, 25mW laser output	Red Laser: 660/20, 725/20, 755 LP
	Violet Solid State Diode: 405nm, 40mW laser output	Violet Laser: 450/50, 550/40

Beckman Coulter (Miami, FL, USA)

Supplementary Table 2. Antibodies used in this study

Antigen	Manufacturer	Catalogue #	Clone	Fluorochrome	Isotype
CD10	BD Biosciences	341112	HI10a	PC7	IgG1 Mouse
CD10	Beckman Coulter	IM3608U	ALB1	ECD	IgG1 Mouse
CD19	Beckman Coulter	A86355	J3-119	PB	IgG1 Mouse
CD27	Beckman Coulter	B12701	1A4CD27	APCA750	IgG1 Mouse
CD27	BD Biosciences	340424	L128	FITC	IgG1 Mouse
CD34	Beckman Coulter	A89309	581	APCA750	IgG1 Mouse
CD34	Beckman Coulter	A86354	581	APCA700	IgG1 Mouse
CD38	Beckman Coulter	A70205	LS198-4-3	PC5.5	IgG1 Mouse
CD45	Beckman Coulter	A96416	J.33	KO	IgG1 Mouse
CD56	Beckman Coulter	A51078	N901 (NKH-1)	PC7	IgG1 Mouse
CD138	Beckman Coulter	A87787	B-A38	APC	IgG1 Mouse
IgD	BD Biosciences	562024	IA6-2	PE	IgG_{2a} Mouse
IgD	BD Biosciences	561314	IA6-2	PC7	IgG_{2a} Mouse
κ CHAIN	Beckman Coulter	A64828	Polyclonal	FITC	F(ab')2 Rabbit
λ CHAIN	Beckman Coulter	A64827	Polyclonal	PE	F(ab')2 Rabbit

PC7, R-Phycoerythrin-Cyanin 7; ECD, Phycoerythrin-Texas Red; PB, Pacific Blue; APCA750, Allophycocyanin-Alexa Fluor 750; APCA700, Allophycocyanin-Alexa Fluor 700; FITC, Fluorescein Isothiocyanate; PC5.5, R-Phycoerythrin-Cyanin 5.5; KO, Krome Orange; APC, Allophycocyanin; PE, R-Phycoerythrin. Beckman Coulter (Miami, FL, USA), BD Biosciences (San Jose, CA, USA)

6. Correlation between CD117+ myeloma plasma cells and hematopoietic progenitor cells in different categories of patients.

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TITLE

Correlation between CD117+ myeloma plasma cells and hematopoietic progenitor cells in different categories of patients.

RUNNING TITLE

CD117+ plasma cells and HPCs

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Abstract

Background: Multiple myeloma (MM) remains an incurable disease, mostly because of the strong interplay between clonal plasma cells (cPCs) and bone marrow (BM) microenvironment. Multiparameter flow cytometry (MFC) allows the simultaneous study of the cPC immunophenotype and alterations involving other cells in BM, but rarely these data are interpreted as connected. One exception to this habit are previous studies about relationship between cPC CD117 positivity and hematopoietic progenitor cell (HPC) distribution in newly diagnosed patients. Methods: we verified distribution of BM CD34+ HPCs in healthy controls and MGUS patients and various categories of responding/relapsing MM subjects divided accordingly to CD117 positivity. Results: Our data completely agree with precedent reports as regards untreated patients. In group with progression of disease, CD117- patients exhibited a lower CD34+CD19-/CD34+CD19- ratio vs CD117+ subjects. Among CD117- cases, CD34+CD19-/CD34+CD19+ ratio was higher in CD117- New vs CD117- Therapy subjects, as reflected by recorded HPC distribution. No differences emerged comparing CD117+ patients. Conclusions: We demonstrate that response to treatment take place through different forms in CD117- and CD117+ patients, implying a distinct regulation in distribution of CD34+ HPC subsets, which becomes evident comparing untreated and treated CD117- patients, but it is impossible to detect in CD117+ cases.

Introduction

Multiple myeloma (MM) is an incurable neoplastic plasma cell disorder characterized by proliferation of clonal/aberrant malignant plasma cells (cPCs) in bone marrow, and presence of monoclonal immunoglobulin (M-protein) in serum and/or urine, associated with immunodeficiency and related organ or tissue impairment [1-3]. With a median age at diagnosis of 65-70 years [3], MM is a disease affecting mainly elderly subjects. MM is usually preceded by a premalignant PC proliferative stage characterized by asymptomatic M-protein production known as monoclonal gammopathy of undetermined significance (MGUS), which is associated with a rate of progression to multiple myeloma of 1% per year [4-5]. In MM long term control of the disease is still an elusive objective. Despite the dramatic progress in therapeutic approaches, due to the introduction of novel categories of drugs (proteasome inhibitors and immunomodulatory agents) [6], no curative strategies has currently been defined. Even patients undergoing to high-dose therapy (HDT) and autologus stem cell transplantation (ASCT) may experiment relapse [7-8]. This phenomenon is strictly related to the strong interplay between cPCs and bone marrow (BM) microenvironment. Residual cPCs may escape therapeutic effects in bone marrow niches, which has been proved to be able to enhance cPC survival and modulate immune system ability to eradicate malignant cells [3, 9-10]. It has now become clear that, in order to cure MM, targeting BM players other than MM cells, and identifying the role of BM microenvironment in response to therapeutic intervention are necessary. In diagnosis and managing of MM and its preceding condition MGUS, multiparameter

flow cytometry (MFC) plays a key role, allowing enumeration of cPCs, and definition of their immunophenotypic characteristics in comparison with normal/reactive polyclonal plasma cells (nPCs) . Although MFC makes possible the simultaneous study of the immunophenotype of cPCs and the alterations involving other cellular components in the BM microenvironment in the same samples (belonging or not to the immune system) [11-16], rarely these data are used to make a connection between immunophenotypic PC characteristics and modifications in BM populations. This is true also in studies regarding hematopoietic progenitor cell subset (HPC) distribution, which has been shown to be impaired in MM patients at diagnosis and relapse [14-16]. CD117 may be aberrantly expressed on cPCs in MM and MGUS [11-12, 17-18], and positivity for this marker confers a favourable prognosis [12, 17-18]. Schmidt-Hieber et al. [19] hypothesized that CD117 might act as an anchor, favouring the adhesion of cPCs to myeloid precursor-associated BM niches mediated by c-kit ligand expressed by stromal cells [19]; no clear mechanism has been defined yet. Previous studies demonstrated that in newly diagnosed patients, no differences were observed as regards CD34+CD38-/dim fraction, but the ratio between BM CD34+CD19- and CD34+CD19+ progenitors was increased in CD117- patients compared to CD117+ subjects [19]. Despite the recent augmented interest in hematopoietic progenitor cell (HPC) distribution, depending on the debate about the utility of quantification of HPC fractions in grafts [20-22], observations about CD34+CD19- and CD34+CD19+ subsets and related ratio (CD34+CD19-/CD34+CD19+ ratio) were not examined in responding and relapsing patients, and currently it is not known whether differences in CD34+CD19- and CD34+CD19+ (Pro-B) cell fractions are preserved in treated patients or may influence depth of response. In this study, we verified distribution of BM CD34+ HPCs in healthy controls and MGUS patients and various categories of responding/relapsing MM subjects. Moreover, after dividing patients accordingly to CD117 expression or absence on PC surface, we compared differences in percentage of CD34+CD19- HPCs and Pro-B cells to detect a potential mechanism related to influence of CD117 positivity on prognosis.

Materials and Methods

Patients and BM samples

Control specimens consisted of 9 BM samples from patients who were suspected to have a haematological disease and revealed to be non onco-hematological subjects (group Control). These patients have no history of MM, MGUS or lymphoid/myeloid neoplasm. BM samples of 44 patients with MM and 10 patients with MGUS submitted to our laboratory for routine analysis were evaluated by MFC. For every patient clinical chemical and immunological profiles, as well as reference intervals were provided by the U.O. Patologia Clinica – Laboratorio Analisi Cliniche of ARNAS Civico, Palermo (Italy). Disease stage was defined according to Durie-Salmon and ISS staging criteria [23-24]. Response to therapy was defined conforming to Bird et al. [25]. Of MM

samples, 12 were obtained at presentation (group New), 8 from patients with progressive disease (group Progressive), 14 from patients in non complete remission (4 Very Good Partial Response, 6 Partial Response and 4 Stable Disease - group Therapy) and 10 from patients who achieved stringent CR (group Complete). MGUS patients were considered as a separate group (group MGUS). Clinical data and history for MGUS and MM cases were provided by U.O. Oncoematologia of ARNAS Civico, Palermo (Italy). Informed consent procedures and forms were proposed to and approved by the ARNAS Civico Medical Ethics Committee. Written informed consent was given by all subjects in line with the Declaration of Helsinki Protocol. BM samples were collected in EDTA tubes and processed in one hour since collection.

Multiparameter Flow Cytometry

Details about antibodies and instrument are indicated in Table 1 and Table 2 respectively. Specimens were fragmented with a sterile syringe and filtered using a 80μm filter; nucleated cells were enumerated using UniCel® DxH™ 800 Coulter® Cellular Analysis System (Beckman Coulter, Miami, FL, USA) and brought to a final concentration of 10^6cells/100μl with PBS w/o calcium and magnesium (EuroClone, Milan, Italy). To stain surface and intracellular markers, the following combinations of antibodies were used: $Cyt\kappa_{FITC}$/$Cyt\lambda_{PE}$/$CD38_{PC5.5}$/$CD56_{PC7}$/$CD138_{APC}$/$CD27_{APC\text{-}AlexaFluor\ 750}$/$CD19_{PB}$/$CD45_{KO}$; Tube 2, $CD27_{FITC}^{\#}$/$CD56_{PE}$/$CD38_{PC5.5}$/$CD117_{PC7}$/$CD138_{APC}$/$CD34_{APC\text{-}AlexaFluor\ 750}$/$CD19_{PB}$/$CD45_{KO}$ (Cyt, Cytoplasmic; FITC, Fluorescein Isothiocyanate; PE, R-Phycoerythrin; PC5.5, R-Phycoerythrin-Cyanin 5.5; PC7, R-Phycoerythrin-Cyanin 7; APC, Allophycocyanin; PB, Pacific Blue; KO, Krome Orange). All antibodies were purchased from Beckman Coulter (Miami, FL, USA), except for # which was purchased from BD Biosciences (San Jose, CA, USA). For staining of surface markers, 100μl of each sample were incubated with the opportune combinations of antibodies for 15 minutes in the dark. Erythrocytes were lysed adding 1 ml of VersaLyse™ Lysing Solution and incubating tubes for 20 minutes in the dark. For intracellular staining of kappa and lambda light chains, 50μl of sample were washed 5 times with 2 ml of PBS w/o calcium and magnesium (EuroClone, Milan, Italy), and processed with PerFix-nc (Beckman Coulter, Miami, FL, USA) following instructions. Samples were all acquired with Navios™ Flow Cytometry System, data were collected with Navios v1.0 Software (Beckman Coulter, Miami, FL) and then analyzed with Kaluza® Flow Cytometry Analysis Software v1.3 (Beckman Coulter, Miami, FL, USA). Daily testing of instrument was performed as indicated: standardization of light scatter, fluorescence intensity and optimal hydrodynamic focusing instrument settings were verified using Flow-Set Pro Fluorospheres (Beckman Coulter, Miami, FL, USA); compensation matrix for each combination of antibodies was tested with CYTO-COMP™ Cell Kit (Beckman Coulter, Miami, FL, USA); optical alignment and fluidics were checked using Flow-Check Pro Fluorospheres (Beckman Coulter, Miami, FL, USA). In order to identify PCs, a combination of CD38, CD138 and CD45 together with side scatter properties was used; the first

gate was set on CD38 vs CD138 as suggested [11]. Distinction between normal/reactive and clonal plasma cell compartments was performed basing on their most frequent aberrant phenotypes (CD38, CD19, CD27, CD117, CD56 and CD45); results were confirmed by the presence of clonal restriction in population showing the abnormal phenotype, and the absence of restriction in normal PCs [11, 26]. The κ:λ ratio was defined as abnormal if < 0.5 or > 3 [27]. A minimum of 200 events in the plasma cell gate and 500 events in CD34+ gate on the CD34/SSC plot were collected for each tube; in order to reach this result, a total of 200,000-2,000,000 events were acquired. For each marker, an internal negative population present within the sample was used to define gates and sample fluorescence background [28-29]. Results for total PCs and CD34+ HPCs are expressed as percentage of cells out of total acquired cells. Data for cPCs and nPCs are indicated as percentage of cells out of total PCs. Fractions of CD34+CD19- and CD34+CD19+ cells are reported as percentage of cells out of total CD34+ HPCs. CD34+CD19+ Pro B cells were recognized through the available markers accordingly to their recognized immunophenotype [30].

Statistical analysis

Continuous non normal data are expressed as median values (range); normal variables are indicated as mean±SD. Baseline differences between groups were assessed by the chi-square test or Fisher's exact test, as needed for categorical variables (with Bonferroni correction for multiple comparisons). The univariate analysis of variance (ANOVA) was performed for parametric variables, and post hoc analysis with the Tukey's test was used to determine pairwise differences. The Mann–Whitney U-test was used for intragroup analysis. The Kruskal-Wallis statistic test with pairwise comparisons was performed for nonparametric analysis. Data were analyzed by IBM SPSS Software 22 version (IBM Corp., Armonk, NY, USA). All p-values were two-sided and $p<0.05$ was considered statistically significant.

Results

Characteristics of patients and plasma cell analysis

A total of 63 subjects (39 male and 24 female, no intergroup significant differences) were included in this study. Patients in Progressive group were significantly older (75.13±10.06 years) than Complete (61.1±8.85 years, p=0.029) and Therapy (61.21±9.01 years, p=0.017) subjects, while no significant differences emerged with Control (62±5.36 years), MGUS (70.8±7.3 years) and New (63.58±12.8). Autologous stem cell transplantation was performed in 6 Complete, 6 Therapy and 2 Progressive patients at least 12 months before the time of this study (not significant). No statistically significant differences as regards therapeutic regimen was detected. Bortezomib+Dexamethasone based treatment was administered to 5 Therapy and 3 Progressive patients. Thalidomide as monotherapy or in combination with bortezomib was given to 1 Complete and 3 Therapy patients respectively. Lenalidomide or lenalidomide containing regimens were the

treatment of choice in 4 Complete and 1 Progressive subjects, while Pomalidomide was administered to 1 Progressive patient. Finally, a total of 13 patients (5 Complete, 6 Therapy and 2 Progressive) underwent suspension of therapy before for at least 15 days before this study was perfomed. No differences emerged for ISS staging at diagnosis (ISS I, 3 Complete, 3 Therapy and 3 New; ISS II 7 Complete, 4 Therapy, 5 New and 5 Progressive; ISS III 7 Therapy, 4 New and 3 Progressive). Instead as regards Durie-Salmon staging at diagnosis, the cases were distributed as follows: IA, 2 Complete, 4 New and 4 Progressive; IIA, 1 Complete, 5 Therapy, 5 New and 2 Progressive; IIB, 3 Therapy, 2 New and 2 Progressive; IIIA, 6 Complete and 2 Therapy (Complete vs New, p=0.018); IIIB, 1 Complete, 4 Therapy and 1 New.

According to literature, nPCs were always CD117-. In turn, cPCs were CD117+ in 50% (5) of MGUS, 42.86% (6) of Therapy, 59.17 (7) of New and 62.5% (5) of Progressive patients (not significant). Considering each group separately, characteristics of patients were homogeneous comparing CD117- and CD117+ cases, except in group New, in which none of CD117+ patients presented characteristics of ISS III stage (vs 4/5 of CD117- patients, p=0.01), and in group Progressive, in which CD117+ patients were significantly older than CD117- ones (81.20±4.09 vs 65±8.66 years respectively, p=0.01).

Significant differences were detected comparing median percentage of total PCs in New [8.64% (0.26%-60.50%)] and Progressive [6.47% (0.75%-30.29%)] with Control [0.15% (0.01%-1.71%), vs New p<0.0005, vs Progressive p=0.001], Complete [0.38% (0.03%-2.49%), vs New p=0.006, vs Progressive p=0.013] and Therapy [0.48% (0.12%-14.40%), vs New p=0.012 and vs Progressive p=0.029], while no difference was observed with MGUS [1.39% (0.36%-2.40%)]. All PCs in Control and Complete were polyclonal, whereas nPCs were 19.12% (5.24%-82.52%) in MGUS, 29.24% (0.14%-96.12%) in Therapy, 1.18% (0.05%-66.12%) in New (vs Therapy p=0.016), and 1.60% (0.25%-12.88%) in Progressive. As regards cPCs, New showed the most elevated median percentage [98.83% (33.88%-99.95%)] compared with Therapy [70.76% (3.88%-99.86%), p=0.016], but no difference emerged in comparisons with MGUS [80.88% (17.48%-94.76%)] and Progressive [98.40% (87.12%-99.75%)]. As regards proportion of CD117+ cPCs, we observed a trend depicting the highest percentage of CD117+ plasma cells in MGUS and the lowest in Progressive (Figure 1).

Intragroup comparisons between CD117+ and CD117- cases as regards percentages of total plasma cells, nPCs and cPCs are indicated in Table 3.

Relationship between CD117 positive and negative cPCs and CD34+ bone marrow HPCs.

No statistical significant differences emerged comparing percentage of total CD34+ HPCs, CD34+CD19+ cells (out of total CD34+ HPCs) and CD34+CD19- cells (out of total CD34+ cells), as well as CD34+CD19-/CD34+CD19+ ratio, despite the fact that Therapy exhibited the highest CD34+CD19+ fraction and the lowest CD34+CD19- fraction and CD34+CD19-/CD34+CD19+

ratio (Table 4). Performing intragroup comparisons between CD117+ vs CD117- cases, we noticed that Therapy CD117- patients showed a higher percentage of total CD34+ cells compared to Therapy CD117+ patients. In New, CD117+ subjects exhibited a higher percentage of total CD34+ and CD34+CD19+ cells vs CD117- patients; in turn, CD117- cases showed a more extended proportion of CD34+CD19- cells and a higher CD34+CD19-/CD34+CD19+ ratio. The frame seemed to be reversed in Progressive group: CD117+ patients exhibited higher percentages of CD34+CD19- cells, and CD117- presented a higher CD34+CD19+ fraction and a lower CD34+CD19-/CD34+CD19+ ratio (Table 5). Exploring relationship between percentage of CD117+ cPCs and CD34+CD19-/CD34+CD19+ ratio, Sperman's correlation coefficients were -0.718 ($p=0.009$) for New, and 0.952 ($p<0.005$) for Progressive. To further deepen how CD117 positivity or negativity may influence distribution of HPCs, we compared Control and Complete data with results obtained from CD117- and CD117+ (Figure 2A-D) cases. CD117- New cases showed the lowest percentage of total CD34+ BM cells compared to Complete and CD117- Therapy patients. In turn, percentage of CD34+CD19- cells and CD34+CD19-/CD34+CD19+ ratio were higher in CD117- New vs CD117- Therapy subjects, while inverted situation was recorded for CD34+CD19+ cells. When we compared Control, Complete and CD117+ cases, no statistical significant differences were observed.

Discussion

The interaction of cPCs with BM microenvironment is fundamental to ensure development and progression of MM. Destruction of BM homeostasis, by a complex system of direct, autocrine and paracrine interactions between components of the BM microenvironment and cPCs, influences proliferation and triggering of anti-apoptotic mechanisms [3, 10]. Normal hematopoiesis is impaired in MM subjects, with anemia being one of MM characteristic clinical features [8]. Precedent studies demonstrated alterations in BM distribution of HPCs in MM subjects. Compared to healthy donors, a substantial reduction of CD34+ HPC subsets and CD19+CD38+CD34+ Pro-B cells in terms of absolute cell count and proportion of mononuclear cells respectively was described in untreated MM subjects [15]. Similarly, percentage of total CD34+ cells and CD19+CD34+ cells (both defined as proportion of total leukocytes excluding PCs) was proven to be decreased at presentation, and CD19+CD34+ cells also in patients at relapse, but not in patients at plateau/remission, vs normal individuals [14]. Coherently, a more recent report confirmed reduction of percentage of CD34+ HPCs (from whole BM cellularity) and CD34+CD38+CD19+ progenitors (out of total CD34+ HPCs) in BM of MM patients vs healthy controls [16]. In all listed studies, no categorization of patients on the basis cPC immunophenotype was performed. In newly diagnosed patients, divided accordingly to CD117 positivity and negativity, percentage CD34+CD38+CD19+ progenitor cells was reported to be higher in CD117+ vs CD117- cases, leading to a significant decreased CD34+CD19-CD38+/CD34+CD19+CD38+ ratio [19]. On the

basis of precedent reports, we were interested in exploring the relationship between presence/absence of CD117 on cPCs surface and distribution of HPCs into CD34+CD19- and Pro-B subsets in MGUS, newly diagnosed, stringent complete responders, treated (but not complete responder) patients, and subjects with progressive disease. We did not observed significant differences among frequency of CD117 positivity comparing all categories of patients, contradicting previous reports [18]. However this discrepancy could be related to variations in sample size. In New, only CD117- were classified as stage ISS III, while none of CD117+ cases showed association with the most advanced ISS stage. These data, together with the trend exhibited by CD117+ cPCs (the highest percentages recorded in MGUS, decreasing through Therapy and New, to the most reduced fraction observed in Progressive) strongly recalls the association of CD117 negativity with features of a more aggressive disease in MM [17], and supports the hypothesis that CD117+ clones might be deleted during progression [18]. In intragroup comparisons of percentages of total, normal and clonal PCs, we observed that in New CD117+ patients nPCs were significantly higher and cPCs were significantly lower vs New CD117- subjects, accordingly to precedent reports [19]. No significant differences were observed in other groups, but the exact mechanism ruling expansion of cPCs vs nPCs remains to be elucidated. As regards distribution of CD34+ HPCs, we did not recorded general variations in percentage of CD34+ HPCs, and CD34+CD19- and Pro-B cell subsets comparing all groups among them. This is openly conflicting with precedent papers [14-16], but may be easily explained considering three important factors: 1) the studies were conducted following different methods of measuring and expressing fractions of CD34+ HPCs; 2) the statistical analysis was performed through different tests; 3) patients were not divided in groups according to the immunophenotype of cPCs, so we cannot evaluate carefully the impact of CD117 positivity on previously reported data. In fact, carrying on our analysis, we noticed that CD117 has a strong influence on CD34+ HPC distribution both in New and Progressive groups, but with different outcomes. In New our findings are consistent with those described by Schmidt-Hieber et al. [19], with a significant inverse correlation between CD117 positivity and CD34+CD19-/CD34+CD19+ ratio. Instead, the opposite situation was recorded in Progressive, with a significant direct correlation between CD117 and CD34+CD19-/CD34+CD19+ ratio reflected by detected measured fractions of CD34+CD19- and Pro-B cells. However, given the small sample size, confirmation by a larger cohort of patients is recommendable. The most surprising results emerged from analysis of Control and Complete data with measured values for CD117- cases. Total CD34+ HPCs were less expanded in CD117- New vs both Complete and CD117- Therapy subjects, thus reflecting a possible reorganization of BM niches in patients able to respond to treatment. Moreover, significant differences were observed between CD117- New and Therapy patients, with New displaying a more extended CD34+CD19- fraction and a reduced Pro-B population compared to Therapy. This was concretized in a higher CD34+CD19-/CD34+CD19+ ratio in CD117- New cases vs CD117- Therapy patients. In turn,

when we compared Control, Complete and CD117+ subjects, no significant differences emerged. These pieces of information are extremely intriguing, since they clearly demonstrate that response to treatment take place through different forms in CD117- and CD117+ patients. Specifically, it implies a distinct regulation in distribution of CD34+ HPC subsets which clearly rises comparing untreated and treated CD117- patients, but it is impossible to detect in CD117+ cases. Given that alterations in distribution of CD34+ HPC subsets exclusively regards patients unable to reach complete response, it will be interesting to evaluate the influence of other immunophenotypes (not described in this studies) on the ability of CD117- patients to achieve a deeper response. Moreover, a possible future step might be study of an eventual correlation between plasma cell immunophenotypic characteristics and mobilization/graft contents. In conclusion, we confirmed previous trends in CD34+ HPC subset distribution in newly diagnosed patients divided accordingly to CD117 positivity. Moreover, we provided some insights in CD34+ HPC distribution in relapsing patients. We also describe different impact of treatment on CD34+ HPCs in CD117- patients vs CD117+ subjects, thus opening the debate about effect of CD117 on mechanism determining prognosis.

Authors' contribution: Authors declare no conflict of interest. Fanny Pojero, Alessandra Casuccio and Francesco Gervasi designed the research study; Fanny Pojero performed the research; Francesco Di Bassiano contributed clinical information; Alessandra Casuccio and Fanny Pojero analyzed the data; Fanny Pojero, Alessandra Casuccio, Francesco Gervasi, Giuseppina Colonna Romano and Calogero Caruso interpreted the data; Fanny Pojero and Alessandra Casuccio wrote the paper; Francesco Gervasi, Giuseppina Colonna Romano and Calogero Caruso revised the paper; all authors approved the paper.

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Tables

Table 1. Characteristics of the instrument.

Model (manufacturer)	Lasers	Detector Filters
Navios (Beckman Coulter) 10 colors, 3 lasers (5+3+2 configuration)	Blue Solid State Diode: 488nm, 22mW laser output	Forward Scatter: 488/10 Blue Laser: 525/40, 575/30, 620/30, 675/20, 695/30, 755LP
	Red Solid State Diode: 638nm, 25mW laser output	Red Laser: 660/20, 725/20, 755 LP
	Violet Solid State Diode: 405nm, 40mW laser output	Violet Laser: 450/50, 550/40

Beckman Coulter (Miami, FL, USA)

Table 2. Antibodies used in this study

Antigen	Manufacturer	Catalogue #	Clone	Fluorochrome	Isotype
CD19	Beckman Coulter	A86355	J3-119	PB	IgG1 Mouse
CD27	Beckman Coulter	B12701	1A4CD27	APCA750	IgG1 Mouse
CD27	BD Biosciences	340424	L128	FITC	IgG1 Mouse
CD34	Beckman Coulter	A89309	581	APCA750	IgG1 Mouse
CD38	Beckman Coulter	A70205	LS198-4-3	PC5.5	IgG1 Mouse
CD45	Beckman Coulter	A96416	J.33	KO	IgG1 Mouse
CD56	Beckman Coulter	IM2073U	N901 (NKH-1)	PE	IgG1 Mouse
CD56	Beckman Coulter	A51078	N901 (NKH-1)	PC7	IgG1 Mouse
CD117	Beckman Coulter	IM3698	104D2D1	PC7	IgG1 Mouse
CD138	Beckman Coulter	A87787	B-A38	APC	IgG1 Mouse
κ CHAIN	Beckman Coulter	A64828	Polyclonal	FITC	F(ab')2 Rabbit
λ CHAIN	Beckman Coulter	A64827	Polyclonal	PE	F(ab')2 Rabbit

Catalogue #, catalogue number; PB, Pacific Blue; APCA750, Allophycocyanin-Alexa Fluor 750; FITC, Fluorescein Isothiocyanate; PC5.5, R-Phycoerythrin-Cyanin 5.5; KO, Krome Orange; PE, R-Phycoerythrin; PC7, R-Phycoerythrin-Cyanin 7; APC, Allophycocyanin. Beckman Coulter (Miami, FL, USA), BD Biosciences (San Jose, CA, USA)

Table 3. Intragroup comparisons between CD117+ and CD117- cases for plasma cells

Group		CD117+	CD117-	p value
MGUS	Tot PCs	1.30 (0.71-2.40)	1.48 (0.36-2.08)	NS
	nPCs	19.17 (6.17-82.52)	10.74 (5.24-51.64)	NS
	cPCs	80.83 (17.48-93.83)	89.26 (48.36-94.76)	NS
Therapy	Tot PCs	0.62 (0.14-14.40)	0.41 (0.12-1.15)	NS
	nPCs	25.19 (0.14-70.80)	71.79 (1.23-96.12)	NS
	cPCs	74.82 (29.20-99.86)	28.21 (3.88-98.77)	NS
New	Tot PCs	6.48 (0.26-20.19)	13.03 (0.44-60.50)	NS
	nPCs	1.68 (0.75-66.12)	0.32 (0.05-1.32)	0.01
	cPCs	98.32 (33.88-99.25)	99.68 (98.68-99.95)	0.01
Progressive	Tot PCs	2.14 (0.75-30.29)	9.03 (8.11-23.98)	NS
	nPCs	6.38 (0.59-12.88)	0.32 (0.25-1.67)	NS
	cPCs	93.62 (87.12-99.41)	99.68 (98.33-99.75)	NS

Results are expressed as median percentage (range). Tot PCs, total plasma cells; nPCs, normal/reactive polyclonal plasma cells (out of total plasma cells); cPCs, clonal/aberrant plasma cells (out of total plasma cells); NS, not significant

Table 4. Percentages of total CD34+ cells and CD34+ fractions

	Tot CD34+	CD34+CD19-	CD34+CD19+	Ratio
Control	1.17 (0.72-2.17)	85.74 (72.94-90.62)	14.26 (9.38-27.06)	6.01 (2.70-9.66)
MGUS	1.34 (0.30-2.46)	88.51 (82.58-97.99)	11.49 (2.01-17.42)	7.82 (4.74-48.75)
Complete	1.98 (0.45-8.26)	88.10 (24.18-98.75)	11.91 (1.25-75.97)	18.75 (0.32-79)
Therapy	1.28 (0.14-2.84)	73.12 (33.91-98.71)	26.88 (1.29-66.09)	2.73 (0.51-76.52)
New	1.26 (0.06-2.61)	87.11 (74.98-98.89)	12.90 (1.11-25.02)	6.77 (3-89.09)
Progressive	0.70 (0.22-0.87)	84.24 (75.73-94.83)	15.76 (5.17-24.27)	5.66 (3.12-18.34)

Results are presented as median values (range). Tot CD34+, total CD34+ hematopoietic progenitor cells; CD34+CD19-, fraction of CD34+CD19- cells (out of total CD34+ cells); CD34+CD19+, fraction of CD34+CD19+ cells (out of total CD34+ cells); Ratio, CD34+CD19-/CD34+CD19+ cellular fraction ratio.

Table 5. Intragroup comparisons between CD117+ and CD117- cases for total CD34+ cells and CD34+ fractions

Group		CD117+	CD117-	p value
MGUS	Tot CD34+	1.47 (0.50-2.46)	1.19 (0.30-1.63)	NS
	CD34+CD19-	95.49 (82.58-97.99)	86.72 (85.73-96.34)	NS
	CD34+CD19+	4.51 (2.01-17.42)	13.28 (3.66-14.27)	NS
	Ratio	21.17 (4.74-48.75)	6.53 (6.01-26.32)	NS
Therapy	Tot CD34+	0.73 (0.14-2)	1.83 (0.85-2.84)	0.013
	CD34+CD19-	78.71 (33.91-98.71)	70.97 (62.86-91.42)	NS
	CD34+CD19+	21.29 (1.29-66.09)	29.04 (8.58-37.14)	NS
	Ratio	4.27 (0.51-76.52)	2.50 (1.69-10.66)	NS
New	Tot CD34+	1.80 (0.49-2.61)	0.56 (0.06-1.22)	0.018
	CD34+CD19-	82.42 (74.98-87.70)	94.59 (90-98.89)	0.003
	CD34+CD19+	17.58 (12.30-25.02)	5.41 (1.11-10)	0.003
	Ratio	4.69 (3-7.13)	17.48 (9-89.09)	0.003
Progressive	Tot CD34+	0.80 (0.22-0.87)	0.60 (0.23-0.62)	NS
	CD34+CD19-	88.75 (80.79-94.83)	77.88 (75.73-80.77)	0.036
	CD34+CD19+	11.25 (5.17-19.21)	22.12 (19.23-24.27)	0.036
	Ratio	7.89 (4.21-18.34)	3.52 (3.12-4.20)	0.036

Results are expressed as median percentage (range). Tot CD34+, total CD34+ hematopoietic progenitor cells; CD34+CD19-, fraction of CD34+CD19- cells (out of total CD34+ cells); CD34+CD19+, fraction of CD34+CD19+ cells (out of total CD34+ cells); Ratio, CD34+CD19-/CD34+CD19+ cellular fraction ratio.

Figures

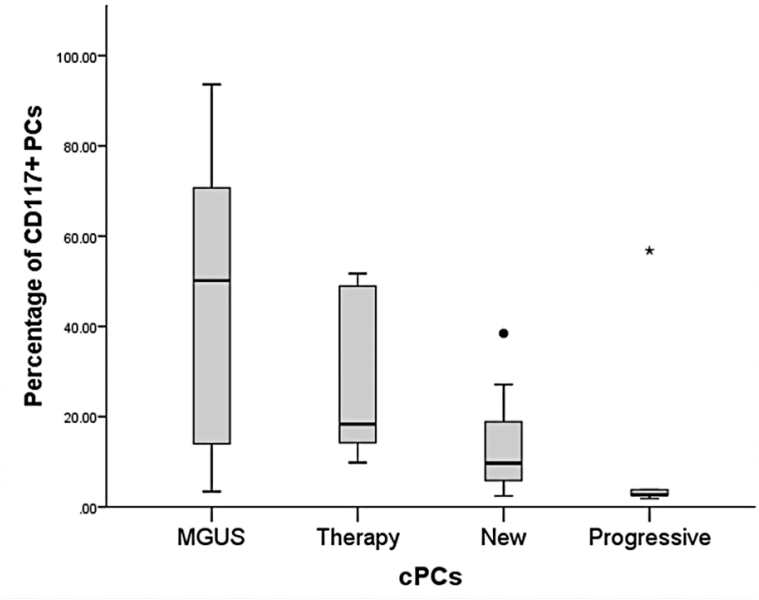

Figure 1. Percentage of CD117+ cPCs. Observed median values (range) were: MGUS 50.15% (3.41%-93.63%); Therapy 18.36% (9.82%-51.69%); New 9.68% (2.44%-38.45%); Progressive 2.67% (1.85%-56.78%). Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. p values of pairwise comparisons are indicated.

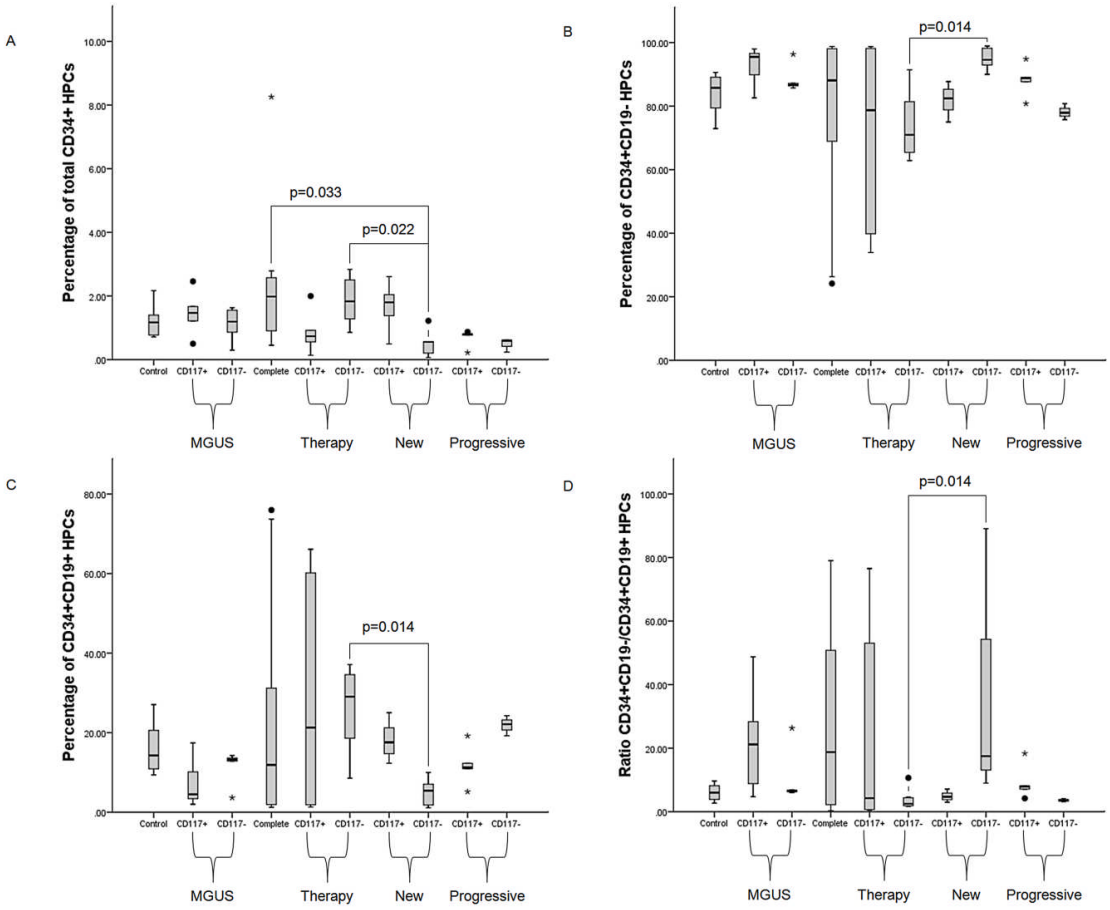

Figure 2. Percentages of CD34+ cells and fractions, and CD34+CD19-/CD34+CD19- ratios for all groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. p values of pairwise comparisons are indicated.

7. Integrated discussion and conclusions.

This is a “getting old” world. The analysis performed by the United Nations [146] has shown that there were 205 million elderly (≥60 years old) persons worldwide in 1950; the proportion has increased to 606 million in 2000. This trend is expected to go on, with the elderly people reaching nearly 2 billion in 2050 [146]. Having their onset usually in advances phases of life, and being monitorable and/or treatable but not curable, MGUS and multiple myeloma represent a challenge both from a clinical and economical point of view [147]. Despite enormous progresses in treatment and MRD detection techniques, disease progression and response cannot be predicted at the time of diagnosis. Beyond the patient stress associated with continue monitoring in both asymptomatic and symptomatic conditions, there is no consensus about therapeutic strategies and establishment of prognosis [148]. Ideally, a laboratory methodology suitable for diagnosis/MRD assessment/prognostic purposes should be widely applicable, offer a good degree of sensitivity, guarantee contained costs, take into account clonal evolution of the diseases, and provide information requiring reasonably short times for manipulation of samples and data elaboration [15, 148]. Among all possible approaches, currently only MFC has the potential of contributing all these advantages at the same time [15, 56, 148]. However, a great reticence in deep application of MFC to various aspects of myeloma and MGUS clinical course may still be met. This depends essentially on absence of specific clonal plasma cell markers, insufficient research on new identification/characterization markers, and most of all discordant results from different reports usually attributable to poor quality design of the studies. As I explained in the introduction, it has already been observed that depth of response may influence outcome, and immunophenotypic variations consequent to drug administration may be observed [28, 49-50, 95-96]. Despite these important premises, a commonly described experimental approach remains based on the study of immunophenotype of clonal plasma cells (and only sometimes polyclonal plasma cells) dividing patients in newly diagnosed subjects and treated/relapsed patients, without considering systematically the effect of treatment, clinical parameters and above all the type of response in rigorous multivariate analysis to detect how all these variables may be related to immunophenotypic characteristics of plasma cells. In addition, an increasing amount of data regarding heterogeneity of normal plasma cell immunophenotype [85, 88-91] produces a discouraging effect on clinicians who have no extended experience in clonal plasma cell identification. Moreover, considering the probability of immunophenotypic changes in normal plasma cells among various categories of patients, and the possibility of using these data to obtain predictive or prognostic information, still represents a completely innovative strategy, although both myeloma and MGUS present a strong component of interaction between neoplastic compartment and surrounding cells [3], including residual polyclonal plasma cells. In the end, the importance of bone marrow microenvironment related characteristics is underrated, and research of a correlation between modifications in other bone marrow populations and type of response and/or immunophenotype is not a common strategy [121-123, 136]. Such a type of investigation would be

particularly useful in order to define patient ability of achieving a good degree of response to therapy, or to start an accurate analysis of immunophenotype related mechanisms which may influence bone marrow cellular composition in last analysis influencing therapy and post ASCT outcome. During my PhD research activity, I kept in mind all these critical points, and I elaborated an accurate, logic and reproducible approach to engage the problem. An essential part of my procedure was the division of patients in clinically defined groups: Control (control subjects), MGUS, Complete (stringent complete responders), Therapy (VGPR, PR and SD patients), New (newly diagnosed subjects), and Progressive (patients presenting progression of disease). As it may be seen in chapter 3, I started my investigation exploring the utility of classical (CD19, CD45, CD117, CD27, CD56, CD20 and CD200) and less analyzed markers (CD30, CD11a, CD49d and CD58) in definition of the "normal" immunophenotype. I clearly demonstrated that normal/reactive plasma cells are not equal among patients, showing a great variability in terms of percentage of positive cells and levels of expression of many antigens. These differences should be carefully considered in elaborating MRD assessment specific panels. In addition, immunophenotypic changes in normal plasma cells showed a degree of association with type of response/relapsing disease, even in the multivariate analysis, thus opening the debate about the introduction of normal plasma cell study in clinical routine both for multiple myeloma and MGUS patients. As regards ranges of expression on normal plasma cells, it can be easily noted that for CD19, CD45 and CD27 percentages of positive polyclonal cells reflect the ranges found in precedent studies (Table 3 of the Introduction) [141]. For CD56, detected percentages essentially did not depart from those described elsewhere, except for an outlier identified in Therapy [141]. However expression close or corresponding to 100% for this antigen has occasionally been reported [87, 91], recalling the hypothesis that CD56+ plasma cells may be artificially low in MFC analysis, acting as an anchor and making plasma cells more resistant to bone marrow aspiration [90]. CD20 was never expressed on plasma cells in Control group, but the range of expression was broad on polyclonal plasma cells in all other groups [141], thus opening the way to a discussion about the role of bone marrow microenvironment (and eventually inflammatory cytokines) on CD20 expression. CD200 was expressed variably on normal plasma cells [141], and my data match with both papers reporting its absence [59, 87] and those indicating positivity [77, 93]. CD58, CD49d and CD11a were never completely absent on polyclonal plasma cells, but a common pattern of expression did not emerged comparing all groups of patients [141]. In turn, frequency of CD30+ cells was modest in both clonal and normal plasmacellular compartments in all groups [141], accordingly to a previous report on myeloma cells [138], so I suggest that CD30 should not be considered for further investigation. Comparing results obtained for normal plasma cells in all groups, significant differences emerged for CD56, CD45, CD27, CD20, CD200, CD11a and CD58 [141]. These data put the accent on the lack of appropriateness of experimental approach considering polyclonal plasma cells as homogeneous in treated and untreated subjects. In addition, described differences

underlie the opportunity of using normal plasma cell analysis in order to determine how changes in bone marrow microenvironment influence surface antigen expression. This approach is particularly important in the look of factors which may be associated with risk of progression or the ability to reach a response to therapy. Deviation from normality in immunophenotype of clonal plasma cells emerged in comparison with plasma cells from non-myeloma and non-MGUS groups, especially for some antigens like CD19 (for MGUS and Progressive), CD56 (for Therapy, New and Progressive), CD117 (for all groups), CD20 (for MGUS and Therapy), CD200 (for Therapy, New and Progressive), CD11a (for New and Progressive) and CD49d (for Progressive) [141]. These alterations always emerged clonal plasma cells vs Control plasma cells, but only in the case of CD19, CD56, CD49d and CD117 the differences with pathological groups interested also Complete group. Therefore, in respect to plasma cells of healthy subjects, plasma cells of stringent complete responders -although polyclonal- present fewer significant immunophenotypic differences from the immunophenotype of clonal plasma cells. Also in distinguishing clonal plasma cells from their normal counterpart, markers showed not to be equivalent; some surface antigens might be successfully employed in all -or almost all- groups (CD45, CD56, CD19, CD117, CD49d), while others reveled their utility in less than three groups (CD27, CD58, CD20 and CD200) [141]. Putting all evidences together, I suggest the use of stage specific MFC panels, with the addition of appropriate MFC markers to the backbone ones (CD38, CD138, CD19, CD45, CD56) [77]; the evaluation of the disease should involve polyclonal plasma cells, in order to follow progressions and explore the opportunity to obtain predictive information. CD49d should be universally present in order to distinguish clonal plasma cells from normal cells; for MGUS diagnosis and monitoring, CD20 and CD200 are suggested to be added; newly diagnosed patients should benefit the addition of CD11a; assessment of disease progression or response to therapy may be made more accurate by the introduction of CD27, CD58, CD11a and CD20 [141]. It is well accepted that in assessing the predictive/prognostic values of MFC markers and their utility in following disease steps, immunological-biochemical-hematological profile must not be ignored, given that it represents the “mirror” of the altered bone marrow microenvironment influencing/being influenced by plasmacellular phenotype [3, 15, 34, 60, 103]. In accordance with this assumption, I elaborated two different statistical models (multinomial logistic regression analysis, and subsequent multivariate regression approach). The objectives were to see whether a correlation exists between the expression of each marker and the attribution of the patients to their own groups, and to determine the effect of other clinical variables on such an eventual correlation. A considerable quantity of significant associations interested polyclonal plasma cells: percentage of cells positive for CD200 and CD56, and levels of expression of CD45, CD19, CD58, CD11a and CD200. In addition, fractions of aberrant plasma cells positive for CD19 and CD200, and intensity of expression of CD19, CD49d, CD117 and CD200 on clonal plasma cells exhibited significance [141]. When the model was elaborated again including significant prognostic variables, I noticed

that percentage of CD200+ normal plasma cells and levels of expression of CD117 on clonal plasma cells and CD19 on normal plasma cells loosed their association, as happened for levels of expression of CD11a in comparing Control vs Complete and MGUS normal plasma cells, MFI ratio for CD200 analyzing Control vs Complete, and percentage of CD200+ clonal plasma cells comparing MGUS vs Progressive [141]. In turn CD19 and CD49d on clonal plasma cells, and CD45, CD58 and CD56 on normal plasma cells maintained their explicative power, so they are good candidates for deeper studies, i.e. analysis of a prospective cohort to examine time and progression dependent fluctuations in levels of MFC markers, in order to define a threshold of expression for each antigen that allows attributing clearly each subject to the relative group [141]. Extending the study with a focus on MGUS and SMM patients and their risk of progression [20] may also be an interesting advancement. The purpose should be the detection of a correlation between the immunophenotypic characteristics of normal and clonal MGUS and SMM plasma cells and the currently used models to estimate the risk of progression in these patients [20, 149]. Assumed this, the look for new markers on both normal and clonal plasma cells is also undelayable in order to ensure the widest applicability of MFC analysis, including samples in which plasma cell detection may be hampered by therapeutic interventions or time and apoptosis dependent loss of antigens [97-98, 101-102]. Their reported medullary patterns of expression, with the actual data about their presence on myeloma plasma cells, constitute the reasons which induced me to select CD229, CD319 and CD54, as possible candidates for suitable alternatives to classical plasma cell selection markers CD38 and CD138. In my experiments, CD229 was persistently positive on both bone marrow and peripheral blood plasma cells. Full positivity is preserved in bone marrow samples stained with APC conjugated antibodies, whereas in 1.1% of samples analyzed through PerCP-Cy5.5 conjugate in combination with fixation/permeabilization technique causes an impairment in staining. In turn, CD319 and CD54 were uniformly positive only on bone marrow plasma cells. When used together with CD38 in the initial gate, the three markers allow an efficient identification of plasma cells, and thus may be considered as potent alternatives to CD38 vs CD138, especially in cases particularly prone to loss or downregulation of CD138 [97-98], or in case anti-CD138 therapy [100]. In turn, when CD229, CD319 and CD54 were used in combination with CD138 or SSC, the scenario changed drastically. With CD54/CD319 vs SSC or CD138 gate, the major problem is detection of all clonal plasma cells, since their levels of expression are partially overlapping with other non-plasmacellular populations. On the contrary, although initial plasma cell identification gate was contaminated with lymphocytes and plasmacytoid dendritic cells, exclusion of non-plasmacellular events was more efficient using CD229 vs SSC and CD138 as initial gate when compared with CD54/CD319 vs SSC and CD54/CD319 vs CD138. Given these results, I propose that CD229 should be considered in further studies on patients undergoing to anti-CD38 therapies in order to assess its possible use in clinical settings. In order to deeply dissect CD229 expression in bone marrow, I included some additional antibody panels in my

experimental routine. I noticed that CD229 was absent on monocytes, eosinophils and neutrophils, whereas T, NK and B lymphocytes were positive in both peripheral blood and bone marrow specimens, in line with previous reports [144].

The study of immunophenotypes or distribution of other cell types in multiple myeloma and MGUS samples, with the simultaneous assessment of surface/intracellular marker expression on plasma cells, is an undiscussed advantage of MFC. Besides the look for other markers in plasma cell detection, the immunophenotypic characterization of other cellular populations in bone marrow samples is fundamental in order to define the actors involved in immune disfunction and unsuccessful eradication of the neoplastic clone. In myeloma patients, many factors concur to determine the general immunosuppression: influence of neoplastic cells on bone marrow microenvironment and immune cells, effect of therapy and normal age-related decline of immune functions [119-120, 150]. Considered that B cells share the same ontological bone marrow path of plasma cells and a number of markers -CD229 for example-, it would sound logic to explore deficiencies affecting this compartment. In spite of this, the great majority of efforts focused on the dysfunctions in T cell compartment, DCs and myeloid derived suppressor cells (MDSCs). So I decided to start an investigation concentrating my analysis on different distribution of naïve/transitional and memory B cells in myeloma patients, looking for a possible relationship with the depth of response. Once again, I recurred to the division of patients in distinct clinical groups, as I exposed some lines above in this discussion. In healthy subjects I found a higher percentage of IgD+CD27+ memory cells compared to Therapy and Complete groups. IgD+IgM−CD27+ memory cells appear to play an important role in respiratory mucosal defense via, whereas CD27+IgM+IgD+ memory cells are involved in the response to encapsulated bacteria [151]. My data open the route to speculations about therapy related mechanisms responsible for increased susceptibility to infections, which could be specific of responding patients. Naïve/Transitional cells were expanded in Complete compared to Therapy patients, showing a possible reorganization of B cell compartment in patients who do not show signs of neoplastic clone, or a superior ability of stringent complete responders to modulate inflammation and its resolution, given the documented cytokine and interleukin profiles documented in the elderly naïve/transitional B cells [130]. As regards DN B cells, an increase of this subset in Therapy patients compared to Complete was recorded. This is not surprising, since DN B cells are described as exhausted B lymphocyte population filling the immunological space especially in elderly subjects [127-130]. Differences in percentage of DN B cells may reflect the ability of various categories of patients to reach deep responses. Considering these results, monitoring fluctuations in percentage of Naïve/Transitional and DN B cells may reveal a useful tool in predicting the type of response, although larger cohort studies are necessary to identify a cut-off value in order to distinguish stringent complete from other responding patients. In addition, the study of immunophenotypic profiles of these lymphocytes as regards chemokine receptor would put some light in definition of their role (or

defects) in myeloma. Another interesting finding is that none of the studied patients showed signs of expansion of a clonal B population. This result must be confirmed by deeper molecular studies, however it represents an interesting start point to explore the presence of a relation between B cells and neoplastic clone. My approach was extremely innovative, since studies about alterations of B compartment in bone marrow of myeloma patients classically target variations in B cell progenitors/precursors [121-123] . Although MFC makes possible the simultaneous study of clonal plasma cells and hematopoietic progenitor bone marrow compartment, rarely these data are used to make a connection between immunophenotypic plasma cell characteristics and modifications in bone marrow populations. An exception to this strategy is the report from Schmidt-Hieber et al., demonstrating that differences in CD34+CD19-CD38+/CD34+CD19+CD38+ ratio in newly diagnosed patients accordingly to CD117 plasmacellular positivity and negativity [136]. On the basis of these preliminary data, I chose to explore the relationship between presence/absence of CD117 on clonal plasma cell surface and distribution of HPCs into CD34+CD19- and Pro-B subsets in MGUS and treated/untreated myeloma subjects. CD117+ clonal plasma cells exhibited a trend, with the highest percentages recorded in MGUS, decreasing through Therapy and New, to the most reduced fraction observed in Progressive. In addition, in New, only CD117- were classified as stage ISS III. These results strongly recalls the association of CD117 negativity with features of a more aggressive disease in multiple myeloma [60], and supports the hypothesis that CD117+ clones might be deleted during progression [103]. In New CD117+ patients normal plasma cells were significantly higher and clonal plasma cells were significantly lower vs New CD117- subjects, accordingly to precedent reports [136]. As regards distribution of CD34+ HPCs, I did not record general variations in percentage of CD34+ HPC, and CD34+CD19- and Pro-B cell subsets comparing all groups among them, conflicting with precedent papers [121-123]. Of note, in the cited works, patients were not divided in groups according to the immunophenotype of clonal plasma cells, so evaluate the exact weight of previously reported data is not possible. Carrying on the analysis, I noticed that CD117 has a strong influence on CD34+ HPC distribution both in New and Progressive groups, but with different outcomes. In New my findings are completely coherent with those described by Schmidt-Hieber et al. [136], with a significant inverse correlation between CD117 positivity and CD34+CD19-/CD34+CD19+ ratio. The opposite situation was recorded in Progressive, with a significant direct correlation between CD117 and CD34+CD19-/CD34+CD19+ ratio. The most surprising results emerged from analysis of Control and Complete data with measured values for CD117- cases. Total CD34+ HPCs were less expanded in CD117- New vs both Complete and CD117- Therapy subjects, reflecting a possible reorganization of BM niches in patients able to respond to treatment. Significant differences in CD34+CD19- and CD34+CD19+ distributions in CD117- New and Therapy patients produced a more elevated CD34+CD19-/CD34+CD19+ in CD117- New subjects vs CD117- Therapy cases. When the analysis involved Control, Complete and CD117+ cases, no differences were detected. My report

clearly demonstrates that response to treatment take place through different forms in CD117- and CD117+ patients. Specifically, it implies a different regulation of CD34+ HPC subsets which clearly rises comparing untreated and treated CD117- patients, but it is impossible to detect in CD117+ cases. Given that alterations in distribution of CD34+ HPC subsets exclusively regards patients unable to reach complete response, I reasonably suppose that other clonal plasma cell immunophenotypes (not described in this study) may exert an influence on the ability of CD117- patients to achieve a deeper response. One future step will surely be a profound exam of other CD34+ HPC subsets, and the research of eventual connection of CD34+ HPC distribution with immunophenotypic profiles (other than CD117 positivity) on both clonal and normal plasma cells. Moreover, it will be intriguing to verify how differences in bone marrow HPC subsets influences graft content. In conclusion, I put together many pieces of the same puzzle, describing a serious impact of well designed MFC studies on the understanding of biology of MGUS and multiple myeloma. Clearly the sample sizes are small, and prospective evaluations are recommendable for every aspect dissected in my experimental activity, but the importance of the listed findings is evident. The added value of this investigation is the continue effort in the direction of individualized/personalized medicine, with the adoption of a method which took constantly into account clinical variables and depth of response. The extremely ordered approach and careful data analysis provided new fundamental insights in some myeloma hot points, i.e. definition of normal plasmacellular immunophenotype, identification of new markers useful in diagnosis/MRD assessment, elaboration of responding/relapsing patients specific MFC panels, exploration of response/progression related distribution of the least investigated immune cells, i.e. B lymphocytes, and description of possible mechanism of immunophenotype dependent response to therapy. I confidentially believe that these data may represent the basis for innovative and productive directions of research in future, and may give a strong impulse to development of MFC translational applications to MGUS and multiple myeloma diagnosis and management.

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