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Flow cytometric sensitivity and characteristics of plasma cells in patients with multiple myeloma or its precursor disease: influence of biopsy site and anticoagulation method

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Abstract

Flow cytometry has increasing relevance for prognosis in myeloma and precursor disease (monoclonal gammopathy of unknown significance/smoldering myeloma), yet it has been reported that plasma cell enumeration by flow varies depending on the quality of marrow aspirate and field biopsied in patchy disease. We demonstrated increased sensitivity of flow over immunohistochemistry in abnormal-plasma cell detection in monoclonal gammopathy ($n = 59$)/smoldering myeloma ($n = 87$). We prospectively evaluated treatment-naïve smoldering myeloma ($n = 9$)/myeloma ($n = 11$) patients for the percentage of abnormal plasma cells/total plasma cell compartment, plasma cell viability/infiltration and flow immunophenotype depending on anticoagulant use, biopsy site and pull sequence in uni-and-bilateral bone marrow biopsies and aspirates. We found no statistical difference regarding the percentage of abnormal plasma cells,

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their immunophenotype or number/distribution in marrow samples even when obtained by different sequence in aspirates, or anticoagulants ($p>0.05$). Our results show that plasma cell enumeration and immunophenotyping by flow cytometry is consistent under different conditions in these populations.

Keywords

Smoldering; myeloma; flow; enumeration; plasma cell; risk

Introduction

Emerging data on the treatment of high-risk smoldering multiple myeloma (SMM) has shown improved overall survival and delayed progression to multiple myeloma (MM) [1]. Important risk factors that allow for identification of patients at high risk of progression to symptomatic MM include the serum free light chain ratio, focal lesions using whole body magnetic resonance imaging (MRI) and plasma cell (PC) immunophenotyping [2 – 6]. The Spanish PETHEMA (Programa de Estudio y Tratamiento de las Hemopatías Malignas) group has developed advanced flow cytometric testing that allows for measurement of the percentage of aberrant plasma cells (aPCs) among all PCs within the bone marrow (BM) compartment (aPCs/BMPCs) [7 – 10]. Using this approach they were able to characterize patients with low (4%) and high (72%) risk of transformation after 5 years of follow-up [11]. Importantly, this approach is based on blind aspirate samples. Furthermore, the European Myeloma Network has reported that plasma cell enumeration by flow cytometry varies depending on the quality of aspirate pull and field biopsied [12]. Our study aimed to determine whether the number and immunophenotypic characteristics of aPCs in patients with SMM and MM vary significantly under different conditions. To our knowledge, no prospective study has been designed to assess this. This study has broad implications for the diagnostic and prognostic validity of a single biopsy/aspiration, for the development of flow cytometry (FC)-based minimal residual disease (MRD) assays, and to expand our insights on immunophenotypic homogeneity/heterogeneity, which is of relevance for the development of immunotherapy targeting surface antigens on myeloma cells [13].

Materials and methods

Patients

Per study protocol, bone marrow core biopsies as well as ethylenediaminetetraacetic acid (EDTA) and heparin anticoagulated aspirates from untreated patients with monoclonal gammopathy of undetermined significance (MGUS) ($n = 59$), SMM ($n = 96$) and MM ($n = 11$) were submitted for FC and histological evaluation for the presence of aPCs as part of screening for prospective clinical trials at our institution (NCT01109407, NCT01572480 and NCT01402284). The diagnosis of MGUS, SMM and MM was based upon standard criteria [14]. All patients with MM presented with lytic lesions, and only some had additional end-organ damage. They all had earlier-stage disease, except for one patient who had International Staging System (ISS) stage III disease. In the bilateral study, most patients with SMM (83%) were at high risk for progression to MM as per Spanish PETHEMA group risk

stratification criteria; however, none of them was at high risk when Mayo Criteria were applied (66% intermediate and 33% low risk by Mayo Criteria). All patients signed institutional review board approved informed consents to be screened for eligibility. Heparin anticoagulated aspirates (second aspirate, first submitted for morphology) were studied by FC in 146 untreated patients with MGUS ($n = 59$) and SMM ($n = 87$) to evaluate the sensitivity of FC compared to morphology with immunohistochemistry in detecting the presence of aPCs. In addition, bilateral BM aspirates and core biopsies from 10 untreated patients with SMM ($n = 5$) and MM ($n = 5$) were submitted for FC and histological evaluation to determine the variability in aPC content from site to site in any individual. The first-pull aspirates were anticoagulated with EDTA and second-pull aspirates were anticoagulated with sodium heparin. In order to further evaluate the effect of anticoagulant (EDTA versus heparin) on results, BM core and aspirate samples were obtained unilaterally from an additional 10 untreated patients with SMM ($n = 4$) and MM ($n = 6$), with first- and second-pull aspirates being anticoagulated with sodium heparin and EDTA, respectively. For all samples, core biopsies were performed after obtaining the aspirates. The first-pull aspirate was sent for microscopic and FC evaluation and the second-pull was sent only for FC. Figure 1 shows the study design.

Morphology and immunohistochemistry

Bone marrow core biopsies were fixed in B Plus fixative and decalcified in Rapid-Cal Immuno (both from BBC BioChemical), embedded in paraffin in a Tissue Tek VIP6 processor (Sakura Finetek), and 4 μ m tissue sections were subjected to immunohistochemistry (IHC) with a CD138 antibody (Syndecan 1, B-A38; Cell Marque) on BenchMark Ultra stainers (Ventana Medical Systems). The percentage of PCs out of the total number of marrow elements in the biopsy was estimated by microscopy based upon CD138 expression. Aspirates and marrow core biopsies were examined for morphological evidence of PC clusters, aPCs and fibrosis. If fibrosis was suspected, reticulin staining was performed.

Flow cytometry analysis

Specimens were processed within 12 h of collection. Red blood cells were pre-lysed by incubating with hypertonic ammonium chloride solution (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) for 10 min at room temperature (maintained at 21 – 23 °C) at a ratio of 1:9 (volume of sample:volume of lysing solution), centrifuged at 500g for 5 min and then washed twice with phosphate buffered saline (PBS) to remove cytophilic antibodies before determining cell number. Cells were then stained for 30 min at room temperature with a panel of antibodies against intracellular kappa and lambda (polyclonal rabbit anti-human, F(ab')_2 ; Dako) and surface CD13PE-Cy7 (clone L138), CD19APC (clone SJ25C1), CD20APC-H7 (clone L27), CD27PE (clone L128), CD38v450 (clone HB7), CD45v500 (clone HI30), CD56PE-Cy7 (clone NCAM16.2), CD81FITC (clone JS-81), CD117PE (clone 104D2), CD138 PerCP-Cy5.5 (clone MI15) (BD Biosciences, San Jose, CA) and CD16FITC (clone DJ130c) (Dako, Carpinteria, CA) as previously described [15]. Cellular viability of lysed and washed BM aspirate cells was assessed by staining with the fluorescent dye 7-amino-actinomycin D (7-AAD) for 10 min at room temperature (7-AAD only stains non-viable cells). For intracellular light chain evaluation, cells were stained with

antibodies against surface antigens and then permeabilized with Fix and Perm reagent (Invitrogen, Frederick, MD) followed by incubation with anti-kappa and anti-lambda antibodies or isotype controls. All cells were fixed in 1.0% paraformaldehyde and stored at 4°C for up to 12 h before acquisition. Specimens were acquired using an eight-color multiparametric approach on a three-laser FACSCanto II (BD Biosciences) with DiVa 6.1.1 software and analyzed by FCS Express 3 software (DeNovo Software, Los Angeles, CA). Acquisition was considered adequate when a significant aPC population was detected or a minimum of 2 million cells was acquired. A minimum of 3 million cells was acquired in all bilateral specimens (SMM $n = 5$, MM $n = 5$) and unilateral multi-anti-coagulant specimens (SMM $n = 4$, MM $n = 6$) to allow full assessment of potential differences in the neoplastic populations.

Determination of aPCs/BMPCs was done as previously described [16]. The definitions of normal PCs (nPCs) and aPCs were based upon the recommendations of the European Myeloma Network and are consistent with those employed by PETHEMA [7 – 9,11]. PCs were identified by gating on cells with CD138 positivity and strong CD38 expression. CD45 and light scatter properties were also examined, to exclude debris, doublets and lymphocytes. nPCs were defined based upon a normal pattern of expression of CD19, CD20, CD27, CD38, CD45, CD56, CD81, CD117 and CD138 as well as polyclonal intracellular light chain expression. aPCs were defined based upon a cluster (antigen expression profile) of PCs with an abnormal pattern of expression of CD19, CD20, CD27, CD38, CD45, CD56, CD81, CD117 and/or CD138 as well as monoclonal intracellular light chain expression (Table I, Supplementary Figure 1 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10428194.2014.955020>). The fraction of PCs among all nucleated cells was determined by quantitating the percentage of bright CD38 and CD138 positive cells in an analysis gate devoid of cellular debris.

Hemodilution determination

Determination of the extent of hemodilution was performed as previously described [17]. Quantification of the extent of neutrophil maturation can be performed based upon CD13 and CD16 expression in neutrophils (fully mature neutrophils express brighter CD13 and CD16 than immature neutrophils). Studies of BM core biopsies indicate that aspirates with 30% or fewer immunophenotypically mature neutrophils have minimal to no hemodilution. The presence of 30–60% immunophenotypically mature neutrophils was considered moderately hemodilute, while aspirates with greater than 60% were considered significantly hemodilute.

Statistical analysis

The midpoints of any percentages reported in ranges were used in statistical evaluations. A two-tailed Wilcoxon signed-rank test was used to determine the significance of differences between paired values.

Results

Mean age $\pm$ standard deviation (SD) for patients with MGUS and SMM was 61 ± 11 and 63 ± 10 , respectively. The different isotypes were distributed as follows, for patients with MGUS: immunoglobulin G (IgG) (67.8%), IgA (22%), IgM (1.7%), IgG and IgM (3.4%), IgG and IgA (1.7%), IgA and IgM (1.7%) and kappa serum light chains only (1.7%); and for patients with SMM: IgG (63.2%), IgA (21.8%), IgM (1.1%), IgG and IgM (2.3%), kappa serum light chains only (6.9%), lambda serum light chains only (3.4%) and urine kappa light chains only (1.1%).

Clinical baseline characteristics of patients participating in the bilateral bone marrow study are listed in Supplementary Table 1 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10428194.2014.955020>. Mean age $\pm$ SD for patients with SMM and MM was 60.77 ± 5.35 and 61.09 ± 11.22 , respectively. The different isotypes were distributed as follows, for patients with MM: IgG kappa (55%), IgG lambda (18%), IgA kappa (9%), kappa light chain (9%) and lambda light chain (9%); and for patients with SMM: IgG kappa (55%), IgG lambda (33%) and kappa light chain (12%). In the 146 untreated patients with MGUS ($n = 72$) and SMM ($n = 87$) evaluated by FC on single heparin anticoagulated aspirates, IHC demonstrated a much higher total percentage of BMPCs compared to FC, but FC was a more sensitive method. IHC detected 3 - 10% (median 10%) and 10 - 80% (median 13%) PCs in the BM core biopsy of MGUS and SMM cases, respectively. On the other hand, FC identified 0.01 - 0.83% (median 0.09%) and 0.03 - 23.5% (median 0.6%) PCs in all acquired cells within the concomitant BM aspirate of MGUS and SMM cases, respectively. Despite the lower level of total PCs detected, FC was more sensitive than IHC in recognition of aPCs. In cases of MGUS, FC definitively identified aPCs in 59/59 cases (sensitivity: 100% [95% confidence interval (CI) 93.88 - 100%]), while IHC definitively detected aPCs in only 12/59 (20.3%), with 18/59 (30.0%) specimens classified as suspicious (slight morphological atypia or light chain predominance) (sensitivity 20.34% [95% CI 10.99 - 32.84%]). In cases of SMM, FC detected aPCs in all 87 SMM BM aspirate samples (sensitivity 100% [95% CI 95.81 - 100%]), while IHC definitively detected aPCs in 81/87 (93.1%) of BM samples, with 6/87 (6.9%) classified as suspicious but not definitive (sensitivity 93.1% [95% CI 85.58 - 97.41%]). The lowest detected limit of aPCs using FC was 25×10^{-6} or 0.0025% of all acquired cells. The percentage of total PCs that were aPCs varied, with a range of 8 - 100% (median 70%) and 16 - 100% (median 98%) in cases of MGUS and SMM, respectively (Table II). Fibrosis was not detected in the study specimens.

When assessing bilateral samples, the mean ($\pm$ standard error of the mean [SEM]) difference in estimated percentage of PC infiltration between the right and left core biopsies was 0.030 ± 0.015 . This difference was not clinically or statistically significant ($p = 0.25$) (Table III).

The viability of all nucleated cells was assessed in 60 samples (40 samples from patients 1 - 10 and 20 samples from patients 11 - 20). First-pull aspirate samples anticoagulated with EDTA had decreased viability when compared to second-pull samples anticoagulated with heparin. This result was independent of location: right sided first-pull EDTA versus second-pull heparin aspirate median viability values were 81% vs. 92%, respectively ($p = 0.039$) and

left sided first- versus second-pull aspirate values were 79% vs. 94%, respectively ($p = 0.0020$). This decreased viability in the first pull was no longer present if heparin was used. There was no difference in viability between first-pull samples anticoagulated with heparin and EDTA anticoagulated second-pull samples (94% vs. 94%, $p = 1.00$) (Table IV).

The percentage of PCs among all cells was evaluated in all aspirate samples. Compared to second-pull aspirates, first-pull aspirates had approximately a two-fold increase in the percentage of PCs regardless of location and anticoagulation: median 1.45% vs. 0.66% in right ($p = 0.0078$) and 0.85% vs. 0.37% in left aspirates ($p = 0.049$) for bilaterally obtained samples and 0.74% vs. 0.46% for unilaterally obtained samples ($p = 0.045$). Interestingly, right pulls had increased % PCs/% all nucleated cells versus left pulls, for both EDTA (1.45% vs. 0.85%; $p = 0.1$) and heparin (0.66% vs. 0.37%; $p = 0.009$) samples. The difference in heparin pulls could reflect different aspiration technique or increased PC return with different locations (Table IV).

The degree of peripheral blood contamination was evaluated on bilateral BM aspirate samples. Increasing hemodilution was appreciated with increasing order of aspirate pull. This was also associated with a decrease in percentage of PCs in the aspirate samples (Figures 2 and 3).

The aPCs/BMPCs did not differ significantly based on location, use of EDTA/heparin or order of aspirate pull. For bilateral samples, right first- and second-pull aspirates had median values of 97% ($p = 0.38$) whereas left first- and second-pull aspirate median values were 98% ($p = 1.00$). For unilateral samples, first- and second-pull aspirate median values were 98% ($p = 0.99$) (Table IV). Patient 1 had one sample (left first-pull EDTA) where the percentage of abnormal PCs was lower than 95% whereas for the three other samples was above 95%. This would not be clinically meaningful, since this patient carried a diagnosis of MM. Patient 8 (SMM) had one sample (left first-pull EDTA) with an aPCs/BMPCs value above 95%, being classified as having high risk of progression to MM as per the Spanish criteria, despite the three other samples being below the cut-off at 94%, 90% and 82% aPCs/BMPCs (Figure 3).

Furthermore, we characterized the phenotypic expression of PCs by FC. All samples had similar expression of CD markers. Table V shows the phenotypic expression of patients 1 – 10. Patients 11 – 20 showed similar results (data not shown).

Discussion

Based on genetic characterization of tumor cell DNA/RNA, MM is a massively heterogeneous plasma cell neoplasia [18]. Current diagnostic criteria are based on the demonstration of significant marrow infiltration with clonal PCs in the presence of clinical evidence of end-organ damage [19]. In the past, several strategies have been proposed to attain a cure or at least control the disease: high-intensity treatments, drug combinations, maintenance therapy and targeted agents [20 – 28]. Most recently, the introduction of therapy earlier in the course of MM development from precursor disease has been of particular interest (i.e. treatment of high-risk SMM) [1,29]. In this regard, enumeration of

aPCs/BMPCs is used to identify patients with SMM at highest risk of progression to MM, and is included in the eligibility criteria of early treatment trials [1,29].

In MM, immunophenotypic enumeration of aPCs has demonstrated prognostic value, assisting in predicting progression-free and overall survival, even when compared to standard response criteria (i.e. serum free light chain ratio and immunofixation) [9,30 – 35]. In addition, FC detection of circulating aPCs and differential marker expression are predictors of overall survival [30,32,36]. Remission based on FC has been shown to be a relevant prognostic factor for patients undergoing autologous stem cell transplant [37,38].

It has previously been demonstrated that when compared to first aspirates, the PC content in subsequent marrow aspirates is significantly decreased. In addition, it has been suggested that BM involvement with PBs may vary depending on location and/or order sequence of aspirate pull [12]. To our knowledge, no reports have been published on the preferred use of anticoagulation for PC analysis by FC. If PC enumeration would differ depending on sample location, order of aspirate pull or method of anticoagulation, the clinical diagnosis of high risk SMM and prognosis of patients with MM could be affected. Furthermore, it has been reported that the sensitivity of aPC enumeration by FC varies up to 100-fold depending on the institution, and there is as yet no standard approach for assessment of MRD in MM [39].

In our study of 146 untreated patients with MGUS ($n=72$) and SMM ($n=87$) we found FC to be highly sensitive, with a greater limit of detection, than morphology combined with IHC, despite being limited to the more hemodilute second aspirate. This confirms previous studies demonstrating the superior performance of FC in detecting aPCs, and its utility in diagnosis and monitoring of response to therapy. In order to address the question of whether a single BM sample can deliver a representative assessment of disease in myeloma, i.e. possible impact of patchy involvement in the general patient population, we assessed 60 samples from 20 patients for differences in PC enumeration and immunophenotype depending on biopsy site.

In addition, the effect of anticoagulant use and order of aspirate pull upon diagnostic results were examined. Our patients had common clinical and diagnostic findings that did not differ from those published in the literature [40]. We have previously shown that there are significant discrepancies between the two clinical models used for risk stratification of patients with SMM [41]. In the present study, none of the patients with SMM were at high risk for progression to MM when assessed by Mayo Clinic Criteria ($n=0/9$), but most patients were at high risk as per Spanish PETHEMA criteria ($n=8/9$). This underscores the need for prospectively validated biological and imaging derived risk factors to better identify an individual patient's risk of progression. As previously reported, morphological evaluation and estimation of plasma cell infiltration by CD138 immunohistochemistry in bilateral BM core biopsies of patients with SMM and MM did not differ significantly [42].

Interestingly, the viability of all nucleated cells in aspirate samples was decreased by approximately 50% when EDTA was used in first-pull aspirates, independent of location. This difference was no longer observed if EDTA was used in second-pull aspirates, perhaps due to the presence of more resilient peripheral blood elements. Viability remained high

when first- and second-pull aspirates were anticoagulated with heparin. It has previously been demonstrated that first-pull aspirates contain a higher number of PCs and myeloblasts, and that these populations decrease with each succeeding pull [12,17]. Heparin has been reported to preserve leukocyte viability better than EDTA, which is in concordance with our results in first-pull samples [43]. Heparin may be superior at preserving the fragile PCs and myeloblasts known to be present in higher numbers in the first aspirate of BM. Based on our results, one could favor using heparin anticoagulation in first-pulls to recover more PCs and preserve viability, which could have implications for the choice of anticoagulation in myeloma MRD studies. In our study, decreased viability did not affect the estimated number, immunophenotypic characteristics or distribution of PCs by FC. Future studies are needed to directly assess PC viability and its relationship to total cell sample viability depending on the method of anticoagulation used.

It has been observed that first-pulls are usually those with the highest quality and contain an increased concentration of PCs. This diminishes with each aspirate as contamination with peripheral blood increases [12]. Our results were concordant with these observations independently of biopsy site and anticoagulation method used (Figures 2 and 3). This difference, however, did not affect clinical interpretation of FC results. Based on our study, if PC recovery is a concern (such as in patients with MGUS or some patients with SMM), one would favor obtaining first-pull aspirates for FC assessment.

Our results show that enumeration of aPCs/BMPCs did not vary significantly either by estimated number or immunophenotypic characteristics of PCs in marrow samples obtained bilaterally. Only one patient with SMM (1/9, 11%) had discordant aPCs/BMPCs in one of the four samples taken. Of importance, the differences observed in viability or decreased number of PCs with successive aspirate pulls did not affect the clinical information derived from FC. Although the overall number of patients enrolled was small, it included patients with both SMM and MM and variability was minimal.

Our findings of limited immunophenotypic variability in bilateral BM aspirates may have major implications in the future. Recent sequencing studies show widespread genetic heterogeneity in MM. For example, frequent mutations in K-RAS (particularly in previously treated patients), NRAS, BRAF, FAM46C, TP53 and DIS3 (particularly in non-hyperdiploid MM) were identified in a series of 203 patients with MM assessed by massively parallel sequencing [19]. In that study, mutations were often present in subclonal populations, and multiple mutations within the same pathway (e.g. KRAS, NRAS and BRAF) were observed in the same patient. In this context, the observed immunophenotypic homogeneity in our study supports the development of immunotherapy targeting defined antigens on the surface of myeloma cells [13].

In conclusion, our prospective study showed the reproducibility of FC evaluation of MM and SMM patient-samples regardless of aspiration site, first versus second aspirate pulls or anticoagulant used. This is very relevant, since MRD negativity in MM by FC has been shown to translate into longer overall survival [33,44]. Molecular methods for MRD detection in MM (i.e. allele-specific oligonucleotide real-time quantitative polymerase chain reaction [ASO RQ-PCR]) have been shown to be slightly more sensitive for MRD detection,

albeit lack consistent applicability across all patients, making MRD assessment by FC the most feasible option for patients with myeloma at the present time [45]. Although we did not appreciate immunophenotypic differences while assessing conventional bilateral iliac crest bone marrow biopsies, directed tumor sampling (such as in plasmacytomas) and advanced molecular profiling may uncover biological variations that may not be readily apparent by FC. Demonstration of the reliability of FC, however, is crucial given the potential utilization of this diagnostic tool in determining prognosis, measuring clinical outcome, initiation of patient treatment and enrollment in clinical trials. Future studies should be directed toward the standardization of methods used for PC FC enumeration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Study design for bilateral samples and anticoagulation use. Aspirates were obtained before core biopsies and anticoagulated with EDTA or heparin as depicted. FC, flow cytometry; EDTA, ethylenediaminetetraacetic acid.

Figure 2.

(A) Hemodilution of bone marrow aspirate samples as a factor of aspirate pull sequence. First-pull samples show minimal to moderate hemodilution, whereas second-pull samples show moderate to significant hemodilution. (B) Median percentage of plasma cells with respect to all nucleated cells in the sample as a factor of hemodilution (minimal = 1.1%, moderate = 0.85%, significant = 0.06%). The difference in median percentage of plasma cells in minimally versus significantly hemodiluted samples is statistically significant (p -value = 0.03, Mann-Whitney test).

Figure 3. Percentage of abnormal plasma cells/all plasma cells within the bone marrow compartment (aPCs/BMPCs) by flow cytometry represented per patient, location and order of aspirate pull. Solid line depicts 95% threshold of aPCs/BMPCs, a risk factor for progression to multiple myeloma according to current risk stratification criteria.

Table 1

Flow cytometric immunophenotyping panel.

Tube	FITC	PE	PerCP	PC7	APC	AH7	V450	v500
S1	CD16	CD19	CD3	CD13	CD34	CD14	CD56	CD45
B8	CD81	CD27	CD138	CD56	CD19	CD20	CD38	CD45
R2	—	CD117	CD19	—	CD138	—	CD38	CD45
B9 ic control	Control	Control	CD138	CD56	CD19	CD20	CD38	CD45
B9 ic K/L	Kappa	Lambda	CD138	CD56	CD19	CD20	CD38	CD45

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll complex protein; APC, allophycocyanin.

Table II

Sensitivity of flow cytometric detection of plasma cells and abnormal plasma cells in MGUS (total 59 cases) and SMM (87 cases).

	PCs as % of total cells	aPCs/BMPCs*	aPCs as % of all cells	Number of aPCs detected
MGUS				
Mean	0.25%	65.2%	0.12%	1094
SD	0.92%	27.2%	0.17%	1054
Median	0.07%	70.0%	0.05%	954
Min	0.01%	8.0%	0.003%	66
Max	0.83%	100.0%	0.74%	5818
SMM				
Mean	1.47%	93.7%	1.44%	3169
SD	2.94%	10.9%	2.92%	4519
Median	0.51%	98.0%	0.50%	2200
Min	0.03%	16.0%	0.02%	299
Max	23.50%	100.0%	23.26%	35 841

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; PCs, plasma cells; aPCs/BMPCs, percentage of abnormal plasma cells in the bone marrow plasma cell compartment; aPCs, abnormal clonal plasma cells.

* Cases of SMM with 95% aPCs/BMPCs were 62 out of 87 cases (71.26%) and cases of MGUS with 95% aPCs/BMPCs were five out of 59 cases (8.47%).

Table III

Estimated percentage of plasma cell infiltration by CD138 immunohistochemistry obtained bilaterally and unilaterally.

Patients	Diagnosis	Core biopsies		Mean difference $\pm$ SEM	p-Value
		Right core	Left core		
Bilateral	1 MM	20–30%	20–30%	–0.030 $\pm$ 0.015	0.25
	2 MM	10%	10%		
	3 MM	<5%	<5%		
	4 MM	60–70%	70–80%		
	5 MM	20–30%	20–30%		
	6 SMM	50%	50%		
	7 SMM	20–40%	30–50%		
	8 SMM	10–20%	10–20%		
	9 SMM	10–20%	10–20%		
	10 SMM	10%	20%		
Unilateral	11 MM	30%			
	12 MM	90–100%			
	13 MM	20–30%			
	14 MM	30–40%			
	15 MM	25%			
	16 MM	5–15%			
	17 SMM	30–40%			
	18 SMM	10–20%			
	19 SMM	60–70%			
	20 SMM	10–15%			

MM, multiple myeloma; SMM, smoldering multiple myeloma; SEM, standard error of the mean.

Table IV

Study outcomes of aPCs/BMPCs, viability and % PCs/% all nucleated cells per patient, location and order of aspirate pull.

Location		1	2	3	4	p-Value								
Viability	Right	First-pull EDTA	92%	82%	78%	89%	82%	71%	74%	80%	80%	61%	81%	0.0039
		Second-pull heparin	91%	96%	88%	92%	92%	92%	94%	95%	92%	91%	92%	
	Left	First-pull EDTA	94%	70%	71%	89%	84%	76%	62%	83%	82%	73%	79%	0.0020
		Second-pull heparin	95%	94%	84%	94%	89%	93%	96%	96%	94%	97%	94%	
			11	12	13	14	15	16	17	18	19	20		
	Unilateral	First-pull heparin	80%	99%	93%	95%	88%	97%	97%	98%	87%	91%	94%	1.00
		Second-pull EDTA	96%	96%	93%	95%	90%	87%	95%	92%	91%	94%	94%	
			1	2	3	4	5	6	7	8	9	10		
	Right	First-pull EDTA	0.60%	0.04%	0.04%	27.00%	8.00%	6.00%	0.60%	1.80%	2.30%	1.10%	1.45%	0.0078
		Second-pull heparin	0.50%	0.12%	0.04%	25.00%	6.00%	3.00%	0.10%	0.80%	1.90%	0.52%	0.66%	
% PCs/% all nucleated cells	Left	First-pull EDTA	0.06%	0.60%	0.16%	19.00%	5.30%	5.30%	1.10%	2.00%	0.60%	0.30%	0.85%	0.049
		Second-pull heparin	0.02%	0.24%	0.02%	17.00%	5.40%	2.00%	0.06%	0.50%	0.90%	0.23%	0.37%	
			11	12	13	14	15	16	17	18	19	20		
	Unilateral	First-pull heparin	3.95%	0.12%	0.49%	41.00%	0.67%	0.95%	0.15%	6.90%	0.80%	0.52%	0.74%	0.045
		Second-pull EDTA	0.41%	0.52%	0.19%	8.40%	0.42%	0.83%	0.26%	2.70%	0.50%	0.25%	0.46%	
			1	2	3	4	5	6	7	8	9	10		
	Right	First-pull EDTA	97%	42%	92%	>99%	97%	>99%	>99%	94%	97%	96%	97%	0.38
		Second-pull heparin	97%	42%	88%	>99%	97%	>99%	>99%	90%	96%	98%	97%	
	Left	First-pull EDTA	93%	42%	91%	>99%	97%	>99%	>99%	96%	98%	98%	97.5%	1.00
		Second-pull heparin	97%	42%	90%	>99%	98%	>99%	>99%	82%	97%	99%	97.5%	
		11	12	13	14	15	16	17	18	19	20			
aPCs/BMPCs	Unilateral	First-pull heparin	>99%	>99%	97%	>99%	93%	97%	97%	>99%	99%	97%	98%	1.00
		Second-pull EDTA	>99%	>99%	96%	>99%	95%	97%	98%	98%	98%	96%	98%	

aPCs/BMPCs, percentage of abnormal plasma cells in the bone marrow plasma cell compartment; PCs, plasma cells; EDTA, ethylene diamine tetraacetic acid.

Table V

Malignant plasma cell immunophenotypic characteristics by patient, location, anticoagulation and order of aspirate pull.

Patient	Location	CD27	CD38	CD138	CD56	CD45	CD20	CD19	CD28	CD81	CD117
1	Right first pull EDTA	32.00%	100.00%	100.00%	100.00%	1.60%	60.20%	0.00%	0.00%	NA	NA
	Right second pull heparin	27.00%	100.00%	100.00%	100.00%	1.00%	52.50%	0.00%	0.00%	NA	NA
	Left first pull EDTA	38.00%	100.00%	100.00%	100.00%	0.70%	51.90%	0.00%	0.00%	NA	NA
	Left second pull heparin	35.00%	100.00%	100.00%	100.00%	0.40%	46.20%	0.00%	0.00%	NA	NA
2	Right first pull EDTA	100.00%	100.00%	100.00%	100.00%	4.90%	0.00%	0.00%	0.00%	NA	NA
	Right second pull heparin	100.00%	100.00%	100.00%	100.00%	5.10%	0.00%	0.00%	0.00%	30.00%	0.00%
	Left first pull EDTA	100.00%	100.00%	100.00%	100.00%	5.40%	0.00%	0.00%	0.00%	NA	NA
	Left second pull heparin	100.00%	100.00%	100.00%	100.00%	1.90%	0.00%	0.00%	0.00%	NA	NA
3	Right first pull EDTA	100.00%	100.00%	100.00%	100.00%	4.50%	31.00%	0.00%	0.00%	NA	NA
	Right second pull heparin	100.00%	100.00%	100.00%	100.00%	5.30%	26.00%	0.00%	0.00%	NA	NA
	Left first pull EDTA	100.00%	100.00%	100.00%	100.00%	4.60%	38.00%	0.00%	0.00%	NA	NA
	Left second pull heparin	100.00%	100.00%	100.00%	100.00%	6.80%	19.00%	0.00%	0.00%	83.30%	0.00%
4	Right first pull EDTA	9.00%	100.00%	100.00%	100.00%	0.00%	3.00%	0.00%	0.00%	NA	NA
	Right second pull heparin	8.00%	100.00%	100.00%	100.00%	0.00%	13.00%	0.00%	0.00%	0.00%	0.00%
	Left first pull EDTA	8.00%	100.00%	100.00%	100.00%	0.00%	13.00%	0.00%	0.00%	NA	NA
	Left second pull heparin	8.00%	100.00%	100.00%	100.00%	0.00%	20.00%	0.00%	0.00%	NA	NA
5	Right first pull EDTA	99.00%	100.00%	100.00%	100.00%	2.00%	2.00%	0.00%	0.00%	NA	NA
	Right second pull heparin	29.00%	100.00%	100.00%	100.00%	0.50%	5.00%	0.00%	0.00%	0.10%	100%
	Left first pull EDTA	99.00%	100.00%	100.00%	100.00%	0.30%	2.00%	0.00%	0.00%	NA	NA
	Left second pull heparin	96.00%	100.00%	100.00%	100.00%	0.70%	1.00%	0.00%	0.00%	NA	NA
6	Right first pull EDTA	100.00%	100.00%	100.00%	100.00%	0.10%	NA	0.00%	0.00%	NA	NA
	Right second pull heparin	100.00%	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	0.00%	NA
	Left first pull EDTA	100.00%	100.00%	100.00%	100.00%	0.00%	NA	0.00%	0.00%	NA	NA
	Left second pull heparin	100.00%	100.00%	100.00%	100.00%	0.20%	0.00%	0.00%	0.00%	NA	NA
7	Right first pull EDTA	36.00%	100.00%	100.00%	0.00%	37.00%	61.00%	0.00%	43.46%	NA	NA
	Right second pull heparin	31.00%	100.00%	100.00%	0.00%	30.00%	48.00%	0.00%	31.01%	60.40%	100%
	Left first pull EDTA	36.00%	100.00%	100.00%	0.00%	32.00%	59.00%	0.00%	40.99%	NA	NA
	Left second pull heparin	21.00%	100.00%	100.00%	0.00%	30.00%	38.00%	0.00%	23.76%	NA	NA

Patient	Location	CD27	CD38	CD138	CD56	CD45	CD20	CD19	CD28	CD81	CD117
8	Right first pull EDTA	21.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	59.40%	NA
	Right second pull heparin	15.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	64.30%	100%
	Left first pull EDTA	23.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	64.80%	NA
	Left second pull heparin	11.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	61.40%	NA
9	Right first pull EDTA	100.00%	100.00%	100.00%	100.00%	0.30%	0.00%	0.00%	NA	42.60%	NA
	Right second pull heparin	100.00%	100.00%	100.00%	100.00%	0.30%	0.00%	0.00%	NA	45.60%	100%
	Left first pull EDTA	100.00%	100.00%	100.00%	100.00%	0.70%	0.00%	0.00%	NA	42.50%	NA
	Left second pull heparin	100.00%	100.00%	100.00%	100.00%	0.60%	0.00%	0.00%	NA	40.70%	NA
10	Right first pull EDTA	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	1.20%	NA
	Right second pull heparin	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	1.30%	100%
	Left first pull EDTA	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	1.30%	NA
	Left second pull heparin	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%	0.00%	NA	1.30%	NA

EDTA, ethylenediaminetetraacetic acid; NA, not available.