Title Page

Title: Polychromatic flow cytometry is more sensitive than microscopy in detecting small monoclonal plasma cell populations.

Running Title: Plasma Cell Flow Cytometry versus Microscopy

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Abstract

Background: There is an emerging role for flow cytometry (FC) in the assessment of small populations of plasma cells (PC). However, FC's utility has been questioned due to consistent underestimation of the percentage of PC compared to microscopy.

Methods: A retrospective study was performed on bone marrow samples analysed by 8-colour FC. Plasma cell populations were classified as polyclonal or monoclonal based on FC analysis. FC findings were compared with microscopy of aspirates, histology and immunohistochemistry of trephine biopsies, and immunofixation (IFX) of serum and/or urine.

Results: FC underestimated PC compared to aspirate and trephine microscopy. The 10% diagnostic cutoff for MM on aspirate microscopy corresponded to a 3.5% cutoff on FC. Abnormal plasma cell morphology by aspirate microscopy and clonality by FC correlated in 229 of 294 cases (78%). However, in 50 cases, FC demonstrated a monoclonal population but microscopy reported no abnormality. In 15 cases, abnormalities were reported by microscopy but not by FC. Clonality assessment by trephine microscopy and FC agreed in 251/280 cases (90%), but all 29 discordant cases were monoclonal by FC and not monoclonal by microscopy. These cases had fewer PC and proportionally more polyclonal PC, and when IFX detected a paraprotein, it had the same light chain as in the PC determined by FC.

Conclusions: FC was more sensitive in detecting monoclonal populations that were small or accompanied by polyclonal PC. This study supports the inclusion of FC in the evaluation of PC, especially in the assessment of small populations.

Introduction

Polychromatic flow cytometry (FC) is used in the assessment of plasma cells (PC) to identify monoclonal populations. PC are identified with CD38, CD45 and CD138 in combination with light scatter properties (1, 2). The two most widely used markers to identify abnormal PC are CD19 and CD56, and the aberrant phenotype of CD19- and/or CD56+ detects at least 90% of cases of neoplastic PC (2). Numerous other markers have been used to identify aberrant PC populations. CD20 is negative on polyclonal PC but may be positive on monoclonal PC (1, 3). Recent reports on minimal residual disease (MRD) detection in multiple myeloma (MM) recommended inclusion of CD27, CD81 and CD117 in addition to CD19 and CD56 (1, 3, 4). Clonality of phenotypically abnormal populations can be readily assessed by intracellular κ and λ light chain (LC) staining (2).

Assessment of PC by FC is useful in the prediction of progression of monoclonal gammopathy of uncertain significance (MGUS) or smouldering MM (SMM) to MM (5). After therapy of MM, assessment of minimal residual disease (MRD) by FC is an independent factor for risk of progression and survival (6, 7). FC has a role in assessment of circulating PC in plasma cell disorders. Enumeration of clonal circulating PC at the time of diagnosis of MM is a predictor of overall survival (8). Recently, the role of FC was reported in the staging of solitary plasmacytoma of bone. The detection of aberrant phenotype plasma cells by FC in a staging bone marrow sample was strongly associated with risk of progression to MM (9, 10). Several of these applications of FC involve assessment of small populations of PC.

Despite numerous reports on the role of FC in the assessment of PC, there has been scepticism surrounding its use, due to the well-known underestimation of PC by FC as

compared to microscopy. Various publications report that FC underestimates PC by 25-60% compared to microscopy (2, 11-13). Plausible explanations for this underestimation include hemodilution of samples sent for FC because of unavailability of first pull bone marrow (BM) aspirates for FC analysis (14), processing artefact due to inherent PC fragility causing loss of gating marker expression (15) and PC micro-aggregation to lipid-enriched spicules and hence loss during cell isolation (11). A recent report demonstrated that second pull aspirates were significantly more hemodilute than first pull aspirates, and consequently the percentage of PC in the first pull was higher than in the second pull (16). Regardless of the cause, concerns about under-estimation of PC numbers by FC may have slowed the implementation of FC in routine assessment of plasma cell disorders.

In the last several years, flow cytometers have been introduced into clinical laboratories that enable assessment of 6-10 markers simultaneously. In the case of PC, it is now possible to combine gating markers (such as CD38, CD45, CD138) with markers that identify abnormal plasma cells (such as CD19 and CD56) and intracellular kappa and lambda LC assessment in a single tube (17, 18). We have carried out a retrospective assessment of the performance of FC using 8-colour analysis in the detection of monoclonal PC populations in BM aspirates. We have compared FC with microscopy of BM aspirates, and with microscopy and immunohistochemistry of the corresponding trephine biopsies, and we have correlated the cellular findings with paraprotein data.

Materials and methods

Samples

A database was constructed on all BM samples investigated for PC markers in the FC laboratory at St Vincent's Hospital (SVH) over a five and a half year period, since the introduction of 8-colour FC. PC investigation was performed in response to relevant

information on the request forms, or when preliminary analysis by FC with CD38 and CD45 detected that over 1% of leukocytes were PC. Normally the first pull of the bone marrow aspirate was committed to microscopy and the second pull to FC. The database included FC (enumeration and immunophenotype), aspirate and trephine microscopy, trephine immunohistochemistry, and immunology (serum and urine electrophoresis (EPG) and immunofixation (IFX)). The vast majority of BM investigations included a trephine biopsy as well as an aspirate, consistent with institutional policy. Data from immunology investigations performed 2 weeks before or after BM analysis were included, and investigations conducted outside this timeframe were discarded. Where data were unavailable for particular investigations, samples were excluded from the analysis of that parameter. When there was more than one sample from the same patient, only the sample with the most complete dataset from all investigations was retained. 42 samples with no evidence of PC and 3 samples with ambiguous clonality were excluded. The project was approved by the St Vincent's Hospital Human Research Ethics Committee.

Flow cytometry analysis

All samples were stained with the following reagents (clone names in brackets): CD45-Pacific Orange or V500 (HI30), CD138-APC (MI15), CD38-Pacific Blue or V450 (HB7), CD19-APC-Cy7 or APC-H7 (SJ25C1), CD20-PerCP (L27), CD56-PE-Cy7 (NCAM16.2), all sourced from Becton-Dickinson, San Jose, CA (BD) except for CD45-Pacific Orange (Invitrogen). Samples were incubated with these antibodies for 10 minutes, then cells were permeabilized with Fix & Perm (Invitrogen), then intracellular κ- and λ-LCs were stained using the cocktail; κ-FITC/λ-PE (1-155-2, TB28-2). All samples throughout the study were stained for the same 8 markers, with the same monoclonal antibodies, and, except for CD19, CD38 and CD45, with the same fluorochromes.

Samples were analysed on a FACSCanto II (BD), that was calibrated using Sphero Rainbow Fluorescent particles 3.0-3.4 μm (BD), and control PB samples were run daily to ensure quality. A stopping gate of 2000 PC and stopping time of 150 seconds was used. Acquisition of 50,000-100,000 total events was typically performed; in some cases 200,000 total events were acquired.

FACSDiva software was used to analyse FC plots and gate populations of interest. Representative dot plots are shown in Figure 1. A 3-marker strategy comprising CD45/CD38/CD138 was used to gate PC. Cursors were set using cell populations within the samples that were not stained by the relevant antibodies. The FC plots were assessed by one of five pathologists for the presence of a monoclonal PC population. All monoclonal populations in the study contained at least 20 events, therefore with a 95% confidence interval, the minimum population detected was 30 events (3). In samples where the minimum 50,000 leukocyte events were collected, the limit of detection was 30/50,000 = 0.06%. In monoclonal populations, if CD19 or CD56 expression was heterogeneous, samples were defined as CD19- or CD56+ if an arbitrarily selected minimum of 30% of PC had that phenotype.

Microscopy and paraprotein detection

The percentage of PC in BM aspirates was calculated after performing a 500 nucleated cell differential count with light microscopy; where a range was given, the mean was taken. The presence or absence of PC with abnormal morphology in aspirates was recorded. For immunohistochemistry, 4 μm sections of trephine biopsies were treated with heat induced epitope retrieval (HIER) and stained using standard protocols on Ventana BenchMark Ultra (Ventana Medical Systems). Antibodies used were CD138 (AbD Serotec clone B-A38) at 1:100, CD56 (Dako clone 123C3) at 1:25, κ (Dako

polyclonal) at 1:6,000 and λ (Dako polyclonal) at 1:6,000. Visualization of antibodies was performed with Ultraview DAB detection and Ultraview Red detection (the latter for lambda with double-stained slides).

Serum and urine EPG were performed using Sebia Hydragel β1-β2 kit (Sebia, Issey-les-Moulineaux, France) and Hydragel 7 HR (Sebia), respectively according to the manufacturer's instructions. Urine underwent routine static concentration prior to EPG (Minicon, Merck Millipore, Darmstadt, Germany). Serum and urine IFX were performed using Hydragel 9 IF (Sebia) according to the manufacturer's instructions.

FC, aspirate microscopy and trephine biopsy reports were made by different pathologists in the Immunology, Haematology and Anatomical Pathology Departments, respectively. The FC was normally reported without knowledge of the other results, but the pathologists reporting microscopy on the aspirates and the trephines normally had access to the final FC report. The paraproteins were reported independently of the other investigations, and paraprotein data was normally available to the pathologists preparing the other reports.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21 software. Descriptive statistics were generated and datasets were assessed for normality using Shapiro-Wilk tests. 2-sample *t*-tests compared means between normally distributed data, and Mann-Whitney U tests compared medians between non-normally distributed data. Chi-square analyses and phi correlations assessed associations between categorical variables. Data were displayed on scatter and box plots. An ANOVA compared means between

multiple groups. Linear regression was used to assess correlation between variables. Statistical significance was achieved with $p<0.05$.

Results

Classification of clonality and monoclonal marker expression

Samples were analysed using the standard PC panel (CD38/CD138/CD45, IC-κ/IC-λ, CD19/CD20/CD56) and assessed by a pathologist for the presence of a monoclonal PC population, which was detected in 212 cases. These cases are referred to as monoclonal, although many of these also contained polyclonal PC. Of the monoclonal samples, 132 were kappa, 78 were lambda and 2 were biclonal kappa and lambda. A monoclonal population was not detected in 105 cases; these cases contained only polyclonal PC and are referred to as polyclonal (Table 1).

Monoclonality was assessed by aberrant marker expression and LC restriction of the aberrant population. 80% of the monoclonal cases were CD19-, 65% were CD56+, and 52% were CD19-/CD56+, which is consistent with previous findings (19-22). CD20, CD38, and CD45 were also used to detect abnormal populations. A case in which CD38 and CD45 were dimmer on monoclonal PC than on accompanying polyclonal PC is shown in Figure 1. 10% of the monoclonal samples were CD20+; although some of these had some features of B cells, these cases were all classified as PC on the basis of microscopy, and were clearly PC on the basis of CD38, CD45 and CD138 analysis by FC. Of the CD20+ cases, 6 were aberrant in both CD19 and CD56, 13 were aberrant in one (either CD19 or CD56) and one case was CD19+/CD56-.

14 (7%) of the monoclonal cases were CD19+/CD56-. In 10 of these, 90-100% of the PC were kappa or lambda, and in the remaining 4, the figure was 78-82%. Further evidence that these populations were indeed monoclonal was provided by other

investigations. All 14 cases had serum paraproteins with LC matching those determined by FC. In the 13 cases with trephine biopsy findings, 12 had a matching LC by immunohistochemistry, and in one case there were no abnormal plasma cells.

In the 212 cases with a monoclonal PC population, 193 had a paraprotein analysis within two weeks of the BM investigation, and 175 (91%) had a detectable paraprotein. In all cases where a paraprotein was detected, it had the same LC as in the PC in FC analysis, except for one case where FC reported a kappa population and IFX reported 2 paraproteins, one kappa and one lambda. Of the 2 cases with biclonal PC by FC, 1 had detectable kappa and lambda paraproteins, but interestingly the other case had no detectable paraprotein.

Polychromatic flow cytometry compared to aspirate morphology

Figure 2 shows that, in nearly all the polyclonal samples, PC were <10% leukocytes by both FC and microscopy of the aspirate, whilst monoclonal cases ranged from little to extensive BM infiltration. Our study confirms well-known observations that the percentage of PC is underestimated by FC compared with microscopy, with FC under-estimating PC percentages in nearly all cases, regardless of clonality. Nevertheless, there was significant correlation between the modalities. The trendline indicated that the 10% threshold for MM by aspirate enumeration corresponded to a 3.5% threshold by FC. Analysis of subsets positive or negative for CD19 or CD20 or CD56 did not reveal a subset with a significant difference in the underestimation of PC by FC, although there was a trend for CD56+ PC to be under-estimated more than CD56- PC, that did not achieve statistical significance (p= 0.059).

Enumeration by FC versus trephine analysis

Trephine microscopy did not routinely report PC percentages, but assigned the abundance of PC to one of 4 categories. Enumeration by FC correlated with trephine analysis, with increasing FC percentages mirrored by trephine descriptors of increased marrow infiltration (Figure 3A). As was the case with aspirate microscopy, FC under-estimated the percentage of bone marrow PC when compared with trephine microscopy. For example, when the trephine reported a moderate increase in PC (10-33% of BM leukocytes), FC reported a median of 2.85% PC. Trephine microscopy also reported a higher abundance of PC compared with aspirate microscopy (Figure 3B). There were 15 cases in which aspirate microscopy reported that PC were less than <10% of leukocytes whereas trephine microscopy reported >10%, including 5 cases where the trephine reported >33%.

Determination of clonality by FC compared with aspirate microscopy

Assessment of clonality by FC was significantly associated with morphological findings on aspirate microscopy. In 81 cases, the PC were polyclonal by FC and normal by microscopy; in 148 cases the PC were monoclonal by FC and abnormal by microscopy. Overall the two modalities correlated in 229/294 (78%) of cases (Table 2). Nevertheless, 50 cases were monoclonal by FC without morphological abnormalities, and 15 cases were polyclonal by FC that did exhibit morphological abnormalities.

The 50 cases that were monoclonal by FC without morphological abnormalities had smaller PC infiltrates, with larger proportions of polyclonal PC, compared to all cases with morphological abnormalities (Table 3). 76% of these populations were CD19-, 57% were CD56+ and 51% were CD19-CD56+, providing supporting evidence for an abnormal plasma cell compartment. EPG results of serum or urine were available for 37 of these 50 cases and a paraprotein was detected in 32 (range 2-26 (median 7)

g/L). In the 32 cases, there were 21 IgG paraproteins, 4 IgA, 4 IgM, 2 Bence Jones proteins, and one case was biclonal IgG and IgA. In all 32 paraproteins, the LCs matched those detected in the PC by FC. Therefore the clonal PC populations were likely to be the sources of the papaproteins, and the populations were sufficiently substantial to produce detectable paraproteins. Of these 50 cases, 19 had no PC abnormalities on trephine microscopy, 27 were monoclonal on the trephine and 4 had no trephine. In the 19 cases with no abnormality on the trephine, FC was the only modality (of the 2 microscopies and FC) to detect an abnormal population. 14 of these 19 cases had a paraprotein, and IFX was not performed in 5 cases. Therefore of the 18 cases with monoclonal PC by flow and no abnormality on aspirate and no paraprotein, only 5 were contained in the group of 19 cases with monoclonal PC by flow and no abnormality on aspirate or trephine.

15 cases were polyclonal by FC but had morphological abnormalities on aspirate microscopy. 11 of these cases were investigated for paraproteins; 10 had a detectable serum paraprotein, and all were IgG, ranging from 2-12 (median 4) g/L. These IgG paraproteins lend support to the significance of the abnormalities on aspirate microscopy. Of these 10 cases with paraproteins, only 1 had fewer than 50 PC detected by FC. Nevertheless, analysis of higher cell numbers may have revealed monoclonal populations. Surprisingly, none of these 15 cases demonstrated a monoclonal PC population by trephine microscopy. In 2 of these cases, the trephine reported a mild increase in PC that was not considered monoclonal. Therefore in these 15 cases, aspirate microscopy was the only modality (of the 2 microscopies and FC) to detect a morphologically abnormal PC population.

Determination of clonality by FC compared with trephine biopsy microscopy

There was a high association between assessment of clonality determined by FC and by immunohistochemical staining of trephine biopsies. The two modalities agreed in 251/280 (90%) of cases (Table 4). Notably, all 29 discrepant cases were reported as monoclonal by FC but polyclonal by trephine microscopy. Dot plots from one of these cases are shown in Figure 1. These 29 cases had smaller PC populations compared with cases identified as monoclonal by trephine microscopy, and contained proportionally larger polyclonal PC populations (Table 5). In most of these cases, the monoclonal cells had an abnormal CD19 and/or CD56 phenotype, the majority (76%) were CD19-, 55% were CD56+, and 34% were CD19-CD56+; only one case was CD19+CD56-.

IFX results were available for 24 of these 29 cases, and in all but 2 cases, paraproteins were detected. In 20 cases, IFX revealed a single serum paraprotein with the same LC as in the PC according to FC, and in one case there were two paraproteins, one kappa and the other lambda, which matched the FC result. In one case that was kappa by FC, 2 paraproteins were detected by IFX, one kappa and the other lambda. 18 of the paraproteins were IgG, 5 were IgA and there was 1 Bence Jones protein; their serum concentration ranged from 2 to 18 (median 4) g/L.

Of these 29 cases, 19 had no abnormality by microscopy of the aspirate. Therefore FC was the only modality (of the 2 microscopies and FC) that detected a monoclonal population in these 19 samples. In the other 10 cases, aspirate microscopy detected increased PC (2.5-10%), and abnormal PC features were reported in 2 of these cases. In the trephine reports on these 10 cases, 6 described PC that were suspicious or atypical or mildly increased with uncertain significance, but monoclonality was not demonstrated. In the remaining 4 cases, the trephine report did not describe any PC abnormality. Of the 29 cases, 26 came from patients who were being assessed for a

plasma cell disorder, mostly following detection of a paraprotein. In one case, a full PC analysis by FC was performed because the initial FC screen found that >1% of BM leukocytes were PC by CD38 and CD45 staining. One case was assessment of treated MM, and one was staging of a solitary plasmacytoma.

Discussion

This study confirmed FC's underestimation of PC percentages compared to microscopy (11-13). Only 10 samples had a higher PC percentage by FC than by aspirate microscopy, and in these cases the overestimation was marginal. The trendline suggests that for the threshold for the diagnosis of MM, a FC value of 3.5% could be similar to the diagnostic criterion of 10% by microscopy used by the International Myeloma Working Group (23). However, with a 3.5% cutoff, some samples that were defined as MGUS by microscopy would become SMM or MM by FC, and *vice versa*. This study was not able to fully corroborate findings by Johnsen et al. (24) that the PC underestimation by FC was more marked in samples that were CD56+, although there was a trend ($p = 0.059$). CD56 expression could be artificially depressed in aspirate specimens used in FC as CD56-mediated adhesion may make CD56+ PC less amenable to aspiration, although this does not explain the enumeration discrepancy between the investigative modalities (25).

Monoclonality by FC was associated with abnormal morphology by aspirate microscopy (26). However there were numerous discrepant samples. There were 50 cases with normal morphology that were monoclonal by FC (Table 2). It is unlikely that these populations were FC artefacts for the following reasons. Firstly, in all cases where paraproteins were identified, the LC of the PC by FC had a matching LC by IFX. Secondly, the majority of these cases had the characteristic monoclonal phenotype of CD19- and/or CD56+. Thirdly, the percentages of PC reported by FC in these 50 cases

were lower, and polyclonal PC were more abundant, compared with the cases where both FC and aspirate microscopy reported abnormalities. Lower percentages of PC and larger proportions of polyclonal PC are likely factors that complicate detection of neoplastic PC by morphology. By contrast, there were 15 cases that were polyclonal by FC with abnormal morphology, and most of these cases had matching paraproteins, suggesting the PC populations were monoclonal. Patchy distribution of neoplastic PC within the BM, or under-representation of PC in the passes sent for FC, or insufficient acquisition of PC could explain the failure of FC to detect these monoclonal populations.

Clonality assessment by trephine and FC were correlated, and there were fewer discordant cases than when aspirate morphology was compared with FC. This is unsurprising given trephine analysis included immunohistochemical LC staining to assess for clonality. Furthermore, more cells are assessed on the trephine than the aspirate, reducing sampling error where patchy infiltration can result in false negatives (27). Additionally, trephine microscopy highlights PC distribution patterns indicative of neoplastic infiltration in the absence of elevated PC percentages or when κ-/λ-LC ratios are unconvincing (28, 29).

Despite these advantages of trephine microscopy, there were 29 monoclonal samples detected by FC but missed by the trephine. In almost all cases, when a paraprotein was detected, its LC matched that of the PC detected by FC, providing evidence that these were significant monoclonal populations. Furthermore, as with the populations not detected by aspirate microscopy, the majority of these cases were CD19- and/or CD56+, providing more evidence that they were neoplastic. These cases had smaller PC populations and larger proportions of polyclonal PC, as was the case with the samples missed by aspirate morphology. These findings suggest that detection of

small monoclonal PC populations by microscopy is difficult, especially in the presence of polyclonal counterparts.

A strength of this study is that the same panel of markers was used to assess PC by FC over several years, enabling accumulation of a large number of samples. A number of limitations of the study should be noted. It is a retrospective study, and in each of the modalities, different pathologists reported on the samples during the study period. The pathologists were not blinded to the other results, although the FC was normally reported before the other modalities. FC did not assess markers such as CD27, CD81, and CD117 which are now included in panels to detect MRD in MM (1, 3, 4). The study has potential implications for detection of small numbers of PC in assessment of MRD, and staging of solitary plasmacytoma of bone, and in assessment of circulating PC. However during the study period, few BM samples were submitted for these purposes, and the study did not assess circulating PC. Instead most of the samples were submitted for assessment of possible MM. Therefore our study does not directly establish the efficacy of FC in MRD, plasmacytoma staging, or circulating PC.

Our finding that FC is more sensitive than microscopy in detecting small monoclonal PC populations is consistent with two recent reports (16, 30) using 8 or 9 colour FC. In one of these reports, FC was shown to be more sensitive than immunohistochemistry for CD138 in detecting abnormal PC in MGUS and SMM (16). In the other report, FC was shown to be more sensitive than microscopy with or without immunohistochemistry in the detection of small PC populations (30). The current report, taken together with these recent publications, indicates the utility of FC using 8 or more colours in the detection of small PC populations.

The findings of superior sensitivity of FC in assessing small PC populations is consistent with the evidence that FC is useful in evaluation of PC in a variety of settings where very low numbers of PC may be clinically important, such as evaluation of MGUS (5) and MRD (6, 7), staging of solitary bone plasmacytomas (9, 10) and assessment of circulating PC as a prognostic factor in MM (8). In these settings, PC may only be present in small numbers, making their analysis virtually impossible by microscopy, and detection of monoclonal PC by microscopy may be further confounded by the presence of polyclonal PC. By contrast, FC can readily analyse very large numbers of cells and can detect PC populations of 0.01% or less, and assessment of monoclonal populations can be determined by aberrant markers as well as LC analysis (12, 31).

This study confirmed the underestimation of PC by FC, but nevertheless showed correlation between microscopy and FC in terms of PC enumeration. Overall, assessment of clonality correlated well between FC and microscopy. However, numerous samples from patients with paraproteins were monoclonal by FC, but were not reported as abnormal by aspirate or trephine microscopy. These results justify inclusion of FC in the assessment of PC disorders.

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Tables

Table 1: Baseline characteristics of BM samples including clonality as determined by FC

Characteristic	Polyclonal	Monoclonal
Total No. (n)	105	212
Median Age (IQR)	64 (19)	67 (17)
Male/Female (n)	72/33	126/86

IQR: Interquartile range

Table 2: Clonality assessed by FC versus morphological abnormalities assessed by aspirate microscopy

		Aspirate microscopy		
		Normal	Abnormal	Total
Flow Cytometry	Polyclonal	81	15	96
	Monoclonal	50	148	198
	Total	131	163	294

There was significant agreement in clonality assessment between the two modalities, chi-square p=0.000.

Table 3: Fewer PC and more polyclonal PC in samples that were monoclonal by FC and normal by aspirate

Characteristic	Monoclonal by FC, Normal by Aspirate Median (IQR)	Abnormal by Aspirate Median (IQR)	Sig. (p)
n	50	163	-
PC (% of BM leukocytes)	0.60 (0.68)	3.3 (10.6)	0.000
Monoclonal: polyclonal ratio by FC	2.6:1 (7.9:1)	16.3:1 (102:1)	0.000

p-values were derived by Mann-Whitney U tests.

Table 4: Clonality assessed by FC versus trephine microscopy and immunohistochemistry

		Trephine microscopy		
		Polyclonal	Monoclonal	Total
Flow Cytometry	Polyclonal	78	0	78
	Monoclonal	29	173	202
	Total	107	173	280

There was significant agreement in clonality assessment between the two modalities, chi-square *p*=0.000.

Table 5: Fewer PC and more polyclonal PC in samples that were monoclonal by FC and polyclonal by trephine

Characteristic	Monoclonal by FC, Polyclonal by Trephine Median (IQR)	Monoclonal by Trephine Median (IQR)	Sig. (*p*)
n	29	173	-
PC (% of BM leukocytes)	0.60 (0.90)	2.8 (10.5)	0.000
Monoclonal: polyclonal ratio by FC	1.7:1 (3.4:1)	17.6:1 (101:1)	0.000

p-values were derived by Mann-Whitney U tests.

Figure Legends

Figure 1

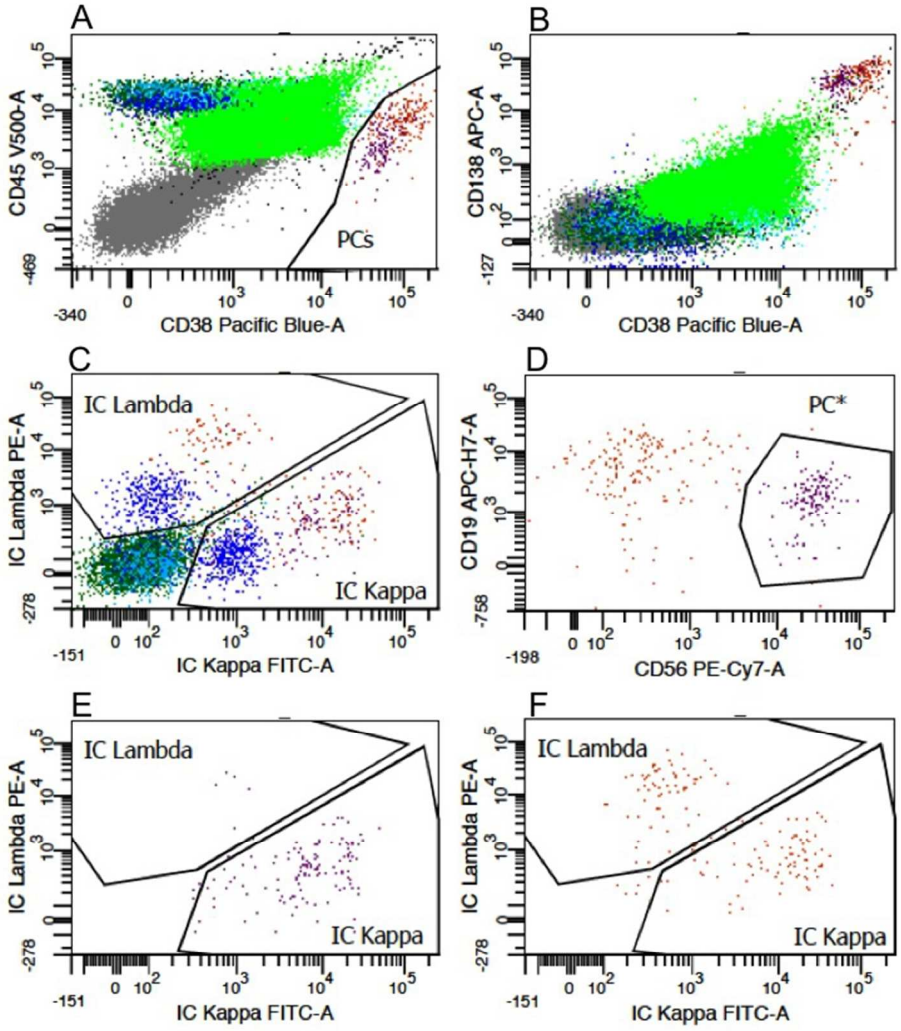
Gating and immunophenotyping of plasma cells. The figure shows dot plots from a bone marrow aspirate containing small numbers of plasma cells (0.4% of total cells by FC). Plasma cells (PC) were gated on the basis of CD38 and CD45 in panel (A), and confirmed to be CD138+ in panel (B). Intracellular (IC) staining with kappa and lambda is shown in panel (C); the blue cells are B cells. Panels (D), (E) and (F) display PC only. In panel (D), CD19dimCD56+ cells (PC*), 131 events, 45% of total PC, are shown in purple. The other plasma cells, largely CD19+CD56-, are shown in red. Only the CD19dimCD56+ plasma cells are shown in panel (E) and these are kappa restricted. Only the other plasma cells are shown in panel (F) and these are polyclonal. Backgating to panel (A) reveals that the monoclonal PC are dimmer for CD38 and CD45 than the polyclonal PC. In this case, the monoclonal population detected by FC was not detected by aspirate microscopy or trephine biopsy immunohistochemistry.

Figure 2

Comparison of plasma cell numbers by FC compared to aspirate microscopy. Data are presented as $\log_{10}$ of PC percentage. The red dotted line shows where the percentage of PC detected by both modalities is the same. The solid black line is the trendline, and the black dotted lines show the 10% threshold for an MM diagnosis by aspirate enumeration, and the equivalent threshold of 3.5% ($10^{0.55}$) for FC. Linear regression showed correlation (R^2= 0.677, p=0.000) between the modalities. The microscopy reports on 15 monoclonal and 36 polyclonal samples did not provide a percentage of PC, and these samples were not included in this analysis.

Figure 3

Comparison of PC numbers by trephine microscopy with flow cytometry in panel (A) and with aspirate microscopy in panel (B). Descriptive categories correspond to the following PC percentages determined by trephine biopsy microscopy: normal/minimal increase (0-2%), mild increase (2-10%), moderate increase (10-33%), significant increase (>33%). The number of cases in each trephine biopsy category is shown (n). The boxes show the median and interquartile range. The upper and lower horizontal lines outside the boxes represent the highest and lowest values that are not outliers. Circles represent outliers (values >1.5 times the interquartile range). The red dotted horizontal reference lines indicate the 10% threshold for MM diagnosis. The trephine microscopy reports on 26 samples did not report a percentage of PC, and these samples were not included in this analysis. Medians were: Panel (A) Normal/minimal increase- 0.4%, mild increase- 0.8%, moderate increase- 2.8%, significant increase- 17.6%; Panel (B) Normal/minimal increase- 2.5%, mild increase- 3.7%, moderate increase- 13.2%, significant increase- 37.0%.



Gating and immunophenotyping of plasma cells.
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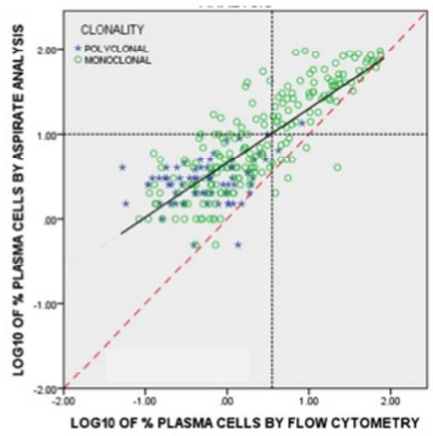

Comparison of plasma cell numbers by FC compared to aspirate microscopy.
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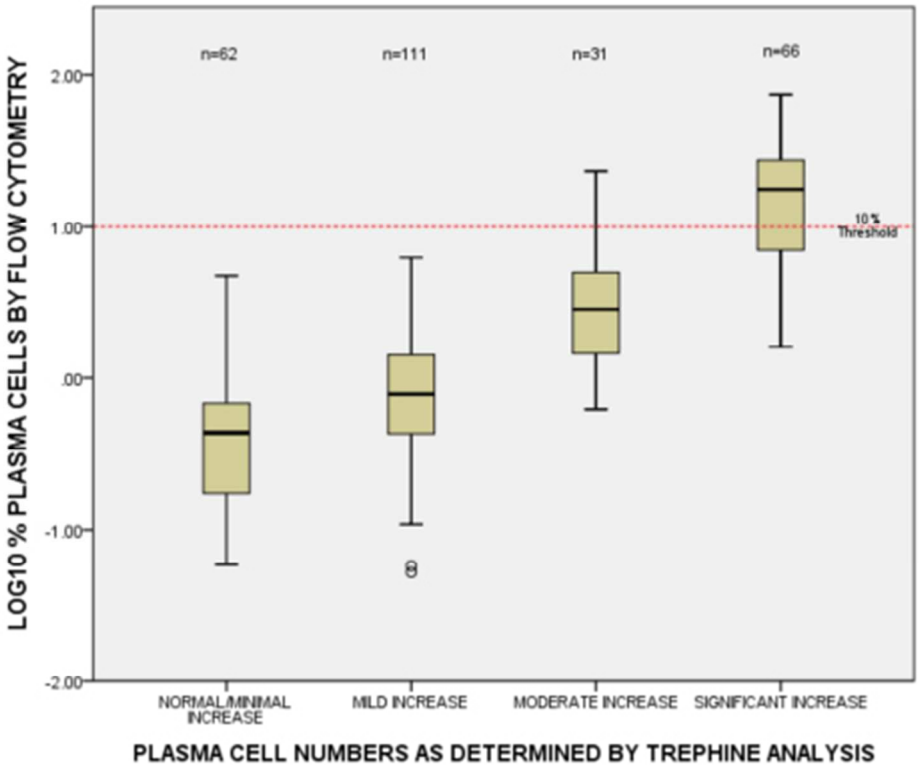

Comparison of PC numbers by trephine microscopy with flow cytometry
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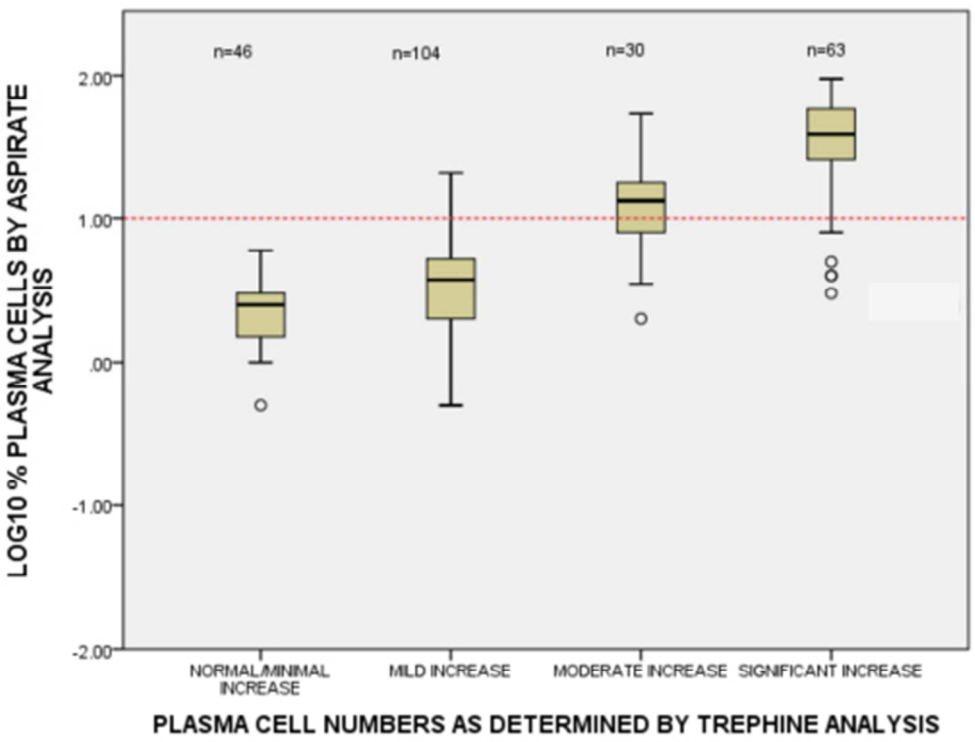

Comparison of PC numbers by trephine microscopy with aspirate microscopy
182x138mm (72 x 72 DPI)