The sensitivity of CD138 immunostaining of bone marrow trephine specimens for quantifying marrow involvement in MGUS and myeloma, including samples with a low percentage of plasma cells

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Accurate quantification of plasma cells in bone marrow samples is essential for the diagnosis, classification and prognosis of plasma-cell dyscrasias. Published comparisons between aspirate/trephine morphology, flow cytometry and immunohistochemistry are lacking. Bone marrow plasma cells from 100 patients with plasma cell myeloma or monoclonal gammopathy of undetermined significance were quantified by a 500-cell differential count on Romanowsky-stained aspirate slides, flow-cytometry gating of $CD38^{bright+}/CD138^{+}$ cells, hematoxylin and eosin trephine section examination and CD138 trephine immunohistology. The results of quantification by the different methods were compared. Compared to other methods, CD138 trephine immunohistology consistently demonstrated greater plasma-cell infiltration. Immunohistology is the most sensitive method for assessment of plasma-cell infiltration at diagnosis or post-therapy, especially in patients with minimal bone marrow involvement.

Key words: myeloma, aspirate, CD138, flow cytometry, immunohistology.

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Accurate quantification of the plasma-cell burden in bone-marrow is essential for diagnosis and assessment of therapeutic response in plasma-cell dyscrasias.[1,2] It has particular importance in defining a prognostically favorable group of patients with multiple myeloma who obtain a *complete response* following high-dose myeloablative therapy.[3] Most laboratories assess the extent of plasma cell infiltration by morphological examination of Romanowsky-stained bone-marrow aspirate samples and hematoxylin and eosin (H&E)-stained trephine sections.[1,4–7] Immunohistological[7–9] and flow cytometric[10] techniques provide additional diagnostic and prognostic information, although their value in plasma-cell quantification has not been systematically assessed against other methods. We compared four methodologies for plasma-cell quantification on bone marrow samples in patients with monoclonal gammopathies of undetermined significance (MGUS) or multiple myeloma to determine i) the degree of correlation between the different methodologies and ii) which was the most sensitive method for plasma-cell quantification.

Design and Methods

One hundred patients with plasma-cell dyscrasias between 2001 and 2005 were retrospectively identified from the Royal Melbourne Hospital bone marrow pathology database. Bone marrow examinations were performed by different people, but conformed to our standard technique protocol over this period.

Bone marrow aspirate samples were taken, with the first 1 mL volume used for smear preparations of five slides, prior to aspiration of a further 5 mL of bone-marrow for immunophenotyping analysis. The most particulate preparations were stained with an International Council for Standardization in Haematology (ICSH) stain containing azure-B and eosin-Y (Figure 1A). The plasma-cell percentage was calculated from a 500-cell differential count, with a denominator including all nucelated cells.

Flow cytometry was performed on bone-marrow aspirate samples collected in lithium-heparin. An EPICS-XL MCL Beckman Coulter Flow-Cytometer was used to identify $CD38^{bright+}$ and $CD138^{+}$ cells ($CD38^{bright+}/CD138^{+}$) which immunophenotypically represent the plasma-cell population. Analysis was continued until a threshold of 5,000 $CD38^{bright+}/CD138^{+}$ events or 200,000 total events was reached, whichever was earlier. The relative proportion of $CD38^{bright+}/CD38^{+}$ cells was calculated against the total nucleated cell population, including nucleated red cells.

H&E and CD138 immunohistochemical staining of bone marrow trephine samples was performed on de-calcified and de-paraffinized sections fixed in B-5 solution. H&E staining was performed on 3 micron thick sections (Figure 1B). Slides were heated with a microwave pressure cooker (Nordic Ware) for 10 minutes to enhance antigen retrieval and then incubated with anti-CD138 (Clone MI15; DAKO). Antibody staining was revealed by DAKO Envision™-peroxidase conjugated to mouse-immunoglobulin and

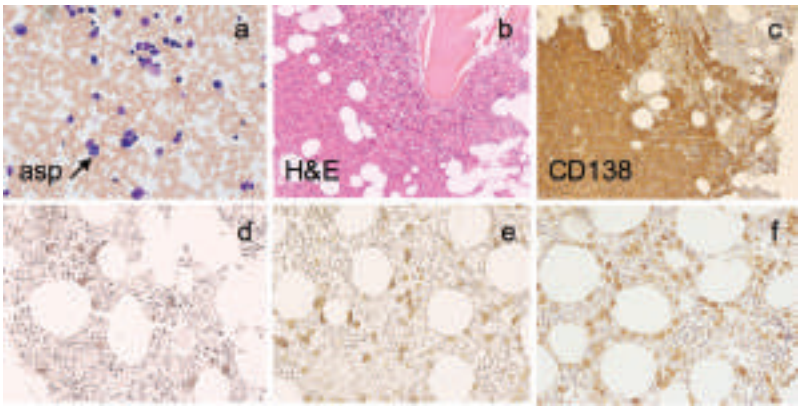

Figure 1. Bone marrow aspirate and trephine plasma-cell enumeration. A-C. Discordant plasma-cell enumeration between (A) bone marrow aspirate smear, (B) trephine H&E stain and (C) trephine CD138 immunohistology. D-F. CD138 Immunohistology for low-burden plasma-cell disease: (D) < 5%, (E) 5-9% (F) 10-20%.

developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB+, DAKO) (Figure 1C). Two independent observers (SJ, AN) estimated the proportion of bone marrow trephine plasma-cell infiltration relative to the total hematopoietic cellularity(11). Infiltration was defined as <5%, 5-10%, 10-15%, 15-20%, 20-30%, etc. (see Figure 1D-F). GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA) was used for descriptive analyses, comparative analyses, and Pearson's correlations, for which Romanowsky-stained aspirate smear plasma-cell counts were taken as the gold standard methodology. Statistical comparison was performed using a two-tailed paired t-test and Bland-Altman analysis.

Results and Discussion

All 100 patients were being investigated for suspected plasma cell neoplasia or re-staging following therapy for multiple myeloma. Forty-nine patients (49%) whose marrow samples were examined had an aspirate plasma-cell count of less than 10%.

Aspirate smear plasma-cell counts were compared to other methodologies

Scatter plots comparing plasma-cell quantification by aspirate counting with flow cytometry, bone-marrow trephine H&E or CD138 immunohistology are shown in Figure 2A-C. Pearson's correlation analysis demonstrated significant correlation of each methodology with aspirate enumeration. Bland-Altman plots comparing percentage difference versus the average plasma cell enumeration of each methodology to aspirate counting are shown in Figure 2D-F. The comparison of plasma-cell quantification for each methodology is shown in Figure 2G. Flow cytometry significantly underestimated bone marrow involvement compared to aspirate smear differential counts (mean difference 13.7, 95% C.I. 10.7 to 16.6, two-tailed paired t-test p=0.0001). In contrast, bone marrow trephine CD138 immunohistology consistently revealed greater numbers of plasma cells in the bone marrow compared to aspirate counting (mean difference 9.3, 95% C.I. 5.0 to 13.5, two-tailed paired t-test p=0.0001)

Estimation by H&E-stained bone-marrow trephine analysis was not statistically different from plasma-cell estimation by aspirate smear (mean difference 2.0, range -6.1 to 2.0, two-tailed paired t-test, p=0.32). Notably, occasional cases with inadequate trephine samples (<0.5 cm length or markedly hypocellular or fibrotic marrow trephines) demonstrated higher aspirate plasma cell counts than did trephine sections stained with H&E or CD138 immunostaining (Figure 2E and Figure 2F).

Bone marrow trephine examination with less than 10% plasma cells by bone marrow aspirate smear

In samples with less than 10% plasma-cell involvement on the Romanowsky-stained aspirate smear, 24% (12/49) of concordant CD138-stained and 8% (4/49) of H&E-stained samples revealed plasma cell infiltration of greater than 10%, with up to 85% plasma-cell involvement in some cases (Figure 2H). Similarly, of patients with less than 5% bone-marrow involvement by aspirate smear examination, 33% (9/27) of CD138-stained and 16% (5/27) of H&E-stained bone marrow trephine samples quantified had greater than 5% plasma cell infiltration of the bone marrow (Figure 2I).

Bone marrow assessment remains integral to the diagnosis, classification and therapeutic monitoring of plasma-cell dyscrasias. Although Romanowsky-stained aspirate smear examination is the traditional and rapid method for plasma cell quantification, it is subject to pre-analytical and analytical variables. These include poorly representative bone marrow aspirate specimens affected by blood dilution, marrow fibrosis or sample clotting. Sampling variability is also encountered, due to focal disease distribution. Representative microscopy field selection for differential counts on samples with patchy plasma-cell distribution on aspirate smears or trephine imprint preparations, is also subject to inter-observer variability.

Importantly, quantification in patients with low percentages of plasma cells on 500-cell aspirate smear differential counts will have a predictably higher coefficient of variation according to a Poisson distribution. The inter-observer coefficient of variation in cases of monoclonal gammopathy of uncertain significance has been demonstrated to be as high as 46%.[12] By comparing various methods of plasma-cell quantification, including flow cytometry and examination of the bone marrow trephine biopsy, our study demonstrated significant differences, especially for bone marrow samples

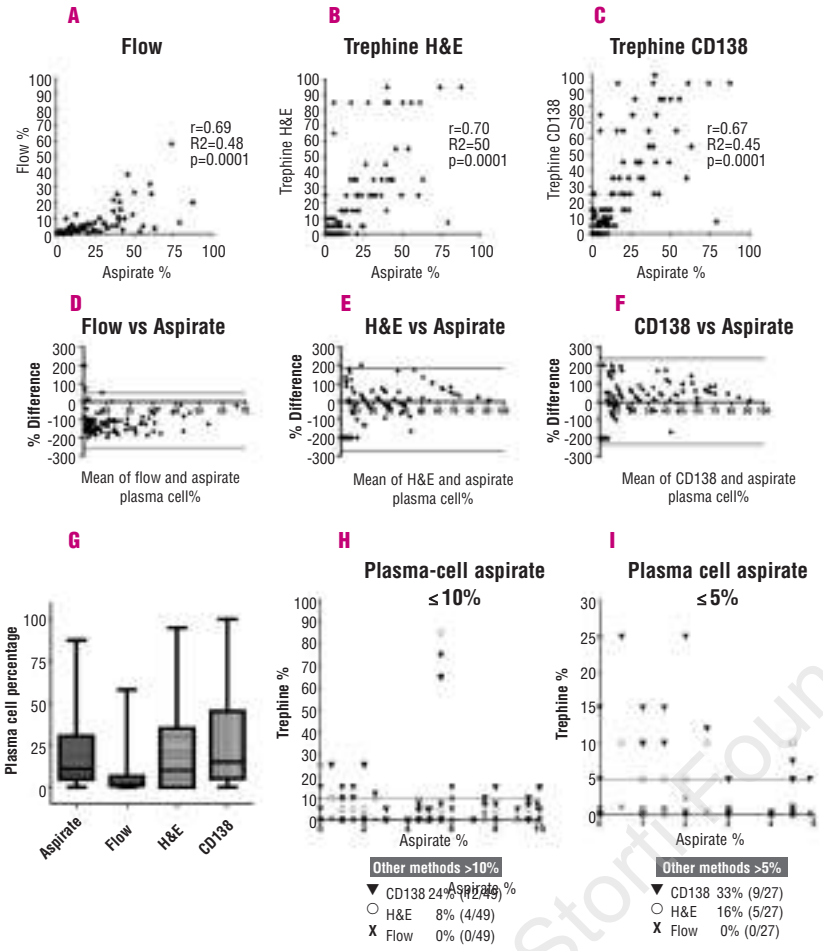

Figure 2. Correlation between aspirate smear and other methods. **A.** Flow cytometry. **B.** Hematoxylin and eosin stain of trephine bone marrow sample. **C.** CD138 Immunohistology of trephine bone marrow sample. **D-F.** Bland-Altman plots of the percentage difference between flow cytometry (**D**), trephine hematoxylin and eosin stain (**E**) and trephine CD138 immunohistochemical stain (**F**) as compared to mean values of plasma cell burden according to a 500-cell aspirate smear count. **G.** Box and whisker plot of plasma-cell quantification by method. **H.** Aspirate smear <10% plasma-cells; comparison with trephine H&E stain, CD138 immunohistology and aspirate flow cytometry. **I.** Aspirate smear <5% plasma-cells; comparison with trephine H&E stain, CD138 immunohistology and aspirate flow cytometry.

with a low percentage of plasma cells. These comprised the majority of samples analyzed, with 49% of samples having less than 10% plasma-cell infiltrate by aspirate smear estimation.

CD138-stained bone marrow trephine samples consistently demonstrated greater plasma-cell infiltration compared to aspirate smear examination, confirming the importance of contemporaneous examination of bone trephine specimens with bone marrow aspirates,[1,4-7] and the value of CD138 immunohistology[7-9] (Figure 2A-C). We note that cytoplasmic staining makes plasma cells appear bigger, and care is needed in the quantitative estimation to take this into account by estimating relative proportions of stained to unstained nucleated cells, rather than evaluating proportional areas of staining. Immunohistochemical nuclear stains, such as the stain for MUM1, may aid plasma cell enumeration in this regard, however, CD138 is more specific for plasma cells, and as such, is more useful, especially for cases in which plasma cells may have lymphoid morphology.[8,13] Reliable immunohistochemical quantification also depends on adequate trephine length with adequate bone-marrow cellularity. Although this was the case for the vast majority of specimens, occasionally, trephine samples of inadequate length, hypocellularity or significant marrow fibrosis, were noted to have lower plasma cell estimates on immunohistology than on aspirate count. Flow cytometry analysis of bone marrow aspirates quantitatively underestimates medullary plasma-cell burden, especially in patients with low burden disease. Pre-analytical variables, such as sample dilution with blood, bone-marrow hypocellularity or fibrosis, sample clotting despite lithium-heparinization and loss of plasma-cell CD138 expression prior to analysis, may have contributed to this finding. Although previous work systematically assessing plasma cell quantification using flow cytometry demonstrated good correlation with morphological assessment, only patients with >15% plasma-cells by bone-marrow morphology criteria had been included.[14] Flow cytometry, however, remains an important tool for the detection of an abnormal plasma-cell phenotype, diagnosis of a malignant plasma-cell clone and detection of minimal residual disease following therapy.[15,16]

No statistical difference between H&E trephine quantification and contemporaneous aspirate smear estimation was found which may be due to the inherent difficulties in identifying small numbers of plasma cells in H&E-stained sections accurately. Significant discrepancies for specific cases were, however, noted between the aspirate and H&E trephine plasma-cell quantification (Figure 2B and 2E), especially when marrow

involvement with myeloma was patchy with or without accompanying marrow fibrosis.

For patients with high levels of disease burden as quantified by aspirate smear examination, H&E-stained trephine sections and CD138 immunohistology are essentially equivalent for diagnostic purposes.

Accurate plasma-cell quantification is critical, especially in patients with a low percentage of plasma cells on bone marrow examination. The WHO and recent IMWG classification of plasma-cell dyscrasias, require 10% or more plasma-cell involvement to reach the diagnosis of multiple myeloma or predict MGUS which is likely to progress.[1,2] In our study, a quarter of patients would have been potentially mis-classified as having less extensive disease by aspirate smear examination alone as compared with concordant CD138-stained trephine samples (Figure 2H).

Similarly, following high-dose myeloablative chemotherapy, the diagnosis of complete response, which carries a more favorable prognosis, requires that there is less then 5% plasma cell involvement (Figure 2I).[3] One third of the patients in our study would have been misclassified as *complete response* by bone marrow criteria using aspirate smear examination alone. A recent publication also suggests that residual disease as assessed by CD138 immunohistology after high dose therapy for myeloma is predictive for earlier disease progression in patients with less than 5% plasma cells on the aspirate, if plasma-cell microaggregates are identified.[9] Thus CD138 immunohistology may allow identification of a group of patients with plasma-cell myeloma at low risk of progression following high dose myeloablative chemotherapy, should a complete response be attained with less than 5% plasma cells and in the absence of plasma-cell microaggregates.

In conclusion, CD138 immunostaining of the trephine bone marrow sample is overall the most sensitive method for quantifying plasma-cell burden, especially in patients with a low percentage of plasma cells on bone marrow aspirate examination. We recommend that trephine CD138 immunohistochemistry be included in routine evaluation along with aspirate smear differential counting and H&E trephine examination to accurately quantify plasma cell involvement of the bone marrow.

AN and SJ are responsible for the whole work, including the conception, design, and conduction of the study, analysis and interpretation of the data and drafting and revising the manuscript. AN interpreted the results and drafted the manuscript. All authors (AN, AW, DB, PC, FF, SJ) were involved in the discussion and revision of the manuscript and gave their permission for the final version submitted for publication. The authors decalre that they have no potential conflicts of interest.

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