TITLE
FLOW CYTOMETRIC DISEASE MONITORING IN MULTIPLE MYELOMA: THE RELATIONSHIP BETWEEN NORMAL AND NEOPLASTIC PLASMA CELLS PREDICTS OUTCOME POST-TRANSPLANTATION.

RUNNING TITLE
PHENOTYPING PREDICTS TRANSPLANT OUTCOME IN MYELOMA

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ABSTRACT

Conventional monitoring strategies for myeloma are not sufficiently sensitive to identify patients likely to benefit from further therapy immediately post-transplant. We have used a sensitive flow cytometry assay that quantitates normal and neoplastic plasma cells to monitor the bone marrow of 45 patients undergoing high dose chemotherapy. Neoplastic plasma cells were detectable at 3 months post-transplant in 42% of patients. Once detected, neoplastic cell levels increased steadily until clinical progression: these patients had a significantly shorter PFS (median 20 months) than those with no detectable disease (median >35 months, P=0.003). Neoplastic plasma cells were detectable in 27% (9/33) of immunofixation-negative complete remission patients. These patients had a significantly shorter PFS than immunofixation-negative patients with no detectable neoplastic plasma cells (P=0.04). Normal plasma cells were present in 89% of patients immediately post-transplant, but were not sustained in most cases. Patients with only normal phenotype plasma cells present at three months post-transplant and also at second assessment had a low risk of disease progression. Patients with neoplastic plasma cells present at three months post transplant, or with only normal plasma cells present at first assessment and only neoplastic plasma cells at second assessment had a significantly higher risk of early disease progression (P<0.0001) with a five year survival of 54% for the high-risk group, compared to 100% in the low-risk group (P=0.036). Analysis of normal and neoplastic plasma cell levels is more sensitive than immunofixation, and can identify which patients may benefit from additional treatment strategies at an early stage post-transplant.

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INTRODUCTION

Many patients who receive high dose therapy (HDT) for myeloma achieve a complete remission by conventional criteria, with a minority achieving a molecular remission. However, with current therapy all patients eventually relapse as a consequence of residual disease. In order to develop effective maintenance strategies, aimed at prolonging the residual disease states, it is important to be able to monitor the behaviour of residual neoplastic plasma cells. In addition to monitoring the malignant cells, it has also been suggested that recovery of normal immunoglobulin levels may be associated with improved outcome. Monitoring the recovery of normal plasma cells may therefore offer an additional approach to predicting the outcome of autologous transplantation.

Current approaches to measurement of residual disease levels are based on morphological assessment of bone marrow biopsies, analysis of the paraprotein levels, or PCR analysis of the immunoglobulin heavy chain VDJ region. Complete remission (CR) is currently defined as the absence of the original monoclonal paraprotein in serum and urine by immunofixation as well as <5% plasma cells in the bone marrow. Using these criteria a number of studies have shown that patients who achieve a complete remission have an improved progression-free and possibly also overall survival compared to partial- or non-responders. However, the difference in survival is insufficient to justify using remission status defined by conventional criteria as a means for adjusting treatment. PCR strategies using primers specific to the neoplastic VDJ region result in sensitivities of up to 1 in 10^6 cells. However, such approaches are not quantitative, are labour-intensive, and only 60-70% of patients
have an amplifiable VDJ region\textsuperscript{6}. Therefore these strategies are difficult to apply in a clinical setting, and it is not clear whether they improve the prediction of outcome post-transplant\textsuperscript{7-9}.

An optimal assay for monitoring residual disease would be robust and universally applicable, and could quantitate low levels of neoplastic plasma cells. We have developed a flow cytometric technique for identifying plasma cells with a sensitivity of 0.01\%. The assay can distinguish neoplastic plasma cells from their normal counterparts based on their CD19 and CD56 expression, even if both cell types are present within the same sample\textsuperscript{10,11}. We have applied this technique to bone marrow aspirates from a series of patients undergoing autologous transplantation in order to determine whether the levels of malignant and normal plasma cells predict outcome after high dose therapy.
METHODS

Patients

Forty-five patients were analysed in this study: 24 male, 21 female, median age at presentation was 55 years (range 41 - 65), β2m levels were <4mg/ml in 49%, 4-8 in 36%, and >8 in 18% of patients. The majority (39/45) were treated in the Medical Research Council Myeloma VII trial high-dose arm. All patients were treated with C-VAMP (continuous intravenous adriamycin and vincristine over 4 days with pulsed intravenous corticosteroids and cyclophosphamide) repeated every three weeks to maximal response. Only patients who achieved at least a partial response to C-VAMP proceeded to high dose therapy, which consisted of melphalan at 200mg/m2 and high dose methylprednisolone with autologous stem cell support. Response was monitored using serum electrophoresis and immunofixation at three monthly intervals. Complete response required less than 5% plasma cells on bone marrow aspirate and biopsy and a negative serum electrophoresis and immunofixation. Bone marrow aspirate samples were obtained at three months post-transplant, and then at three - six monthly intervals thereafter. Samples were analysed prospectively.

Flow cytometry

Leucocytes were prepared by incubation with a tenfold excess of ammonium chloride (8.6 g/L in distilled H₂O) for 5 minutes, and washed twice in FACSFlow (BD Biosciences, Oxford, UK) containing 0.3% Bovine Serum Albumin (BSA, Sigma, UK). 1 x 10⁶ leucocytes were stained with 10µl volumes of each pre-titred antibody per test for 20 minutes at 4°C, washed twice, and acquired using a Becton Dickinson FACSort with CELLQuest v3.1 software (BD Biosciences, Oxford, UK). Cells were
incubated with CD45 FITC (in-house), CD38 PE/Cy5 (in-house), and either CD3 PE (in-house), CD138 PE (Serotec, Oxford, UK), CD19 PE (in-house), or CD56 PE (BD Biosciences, Oxford, UK). Between 50,000 and 500,000 total cells were analysed in each test.

The gating strategy is optimised to exclude contaminating events, particularly B progenitors which are common in samples from patients immediately post-transplant, as well as apoptotic cells and cellular debris. Analysis of CD38 vs CD138 expression

The gating strategy is optimised to exclude contaminating events, particularly B progenitors which are common in samples from patients immediately post-transplant, as well as apoptotic cells and cellular debris. Analysis of CD38 vs CD138 expression
(i) provides the best separation of plasma cells from other leucocytes, but is also subject to contamination with cells binding antibodies non-specifically. This can be detected on the CD38 vs CD45 plot (iii) to the right of the plasma cell population. As such, an initial region (R1) is set around cells expressing a high level of CD38 and CD138 (i), and a second region (R2) set on the light scatter of gated CD38+138+ cells (ii). A third region (R3) was set around the cells satisfying both R1 and R2 for CD38 and CD45 expression (iii). Regions R2 and R3 are then optimised until events falling within both of these regions are all CD138+ and CD3-. Horizontal quadrant markers are set according to the CD3 control for analysis of CD138 and CD19 expression. CD56 expression is weak on normal plasma cells, and the marker is set higher than control (at 100 as standard) as this provides a better discrimination between normal and neoplastic cells. The expression of CD19 PE and CD56 PE is then used to distinguish between normal and neoplastic plasma cells. The former are consistently CD19+56dim whereas the latter are CD19- or CD19+56+\textsuperscript{10,12}. The level of CD19 expression is broad on normal plasma cells, and up to 10% may be CD19- in comparison to control. Therefore, samples were only classified as containing neoplastic plasma cells if more than 10% had an abnormal phenotype. A minimum of 50 events that satisfied the gating strategy (Figure 1) were required for identification of a neoplastic or normal plasma cell population. Up to 500,000 events were acquired, allowing a maximum sensitivity of detection of 0.01% in all cases. Dilute non-representative aspirate samples are a significant problem in all minimal residual disease studies. This assay allows the identification of such samples in most cases. Those containing <0.01% normal or neoplastic plasma cells were always unrepresentative of the trephine biopsy appearance, and were not included in the study. In this series of patients, unsuitable aspirates were received in approximately
6% (3/48) of cases. Marrow aspirate samples containing both neoplastic and normal plasma cells were always representative, as normal CD138+ plasma cells are only found in bone marrow, not in peripheral blood\textsuperscript{10}. All patients with disease on the trephine biopsy had neoplastic plasma cells detectable by flow cytometry, although the degree of infiltration was underestimated in a minority of cases.

**Fluorescent immunoglobulin heavy chain gene (IgH) PCR analysis using consensus primers**

High molecular weight DNA was obtained from separated leucocytes by proteinase K digestion, phenol/chloroform extraction and cold ethanol precipitation. DNA was amplified with a 5' FITC-labeled primer to a consensus region of the JH gene, and a primer to a consensus framework 3 (Fr3) region, or a mixture of primers to consensus framework 1 (Fr1) regions on each of the six VH gene families, as reported previously\textsuperscript{6}. Electrophoresis and analysis was performed using an Applied Biosystems automated DNA sequencer. Electrophoretograms were produced from the fluorescence intensity data, representing the size and relative amount of each PCR product as a peak on a histogram. This PCR assay will detect neoplastic plasma cells at the level of 1 in $10^5$ leucocytes if no other B cells are present. If B cells are present, which is the case in most patient samples, the assay will identify a population that represents more than 2% of total amplifiable B cells, equating to a sensitivity of 1 in $10^3 – 10^4$ total leucocytes\textsuperscript{13}. 
RESULTS

Sensitivity of flow cytometry assay: comparison with consensus-primer IgH-PCR

We have previously demonstrated that fluorescent consensus-primer IgH-PCR has a comparable sensitivity for the detection of residual disease to immunofixation. In order to determine whether flow cytometric assessment would be more applicable, we compared the flow assay to consensus-primer IgH-PCR analysis. Twenty-five patients had amplifiable DNA from presentation marrow samples, of which 16/25 (64%) had an amplifiable IgH rearrangement. This is consistent with previous studies demonstrating an amplifiable rearrangement in up to 80% of patients. The flow assay detected neoplastic plasma cells at presentation in all patients included in this study, and we have previously demonstrated in a series of over 500 patients that the assay will detect neoplastic plasma cells in over 98% of cases.

Thirty-three follow-up samples were available from patients with an amplifiable IgH rearrangement. Neoplastic plasma cells were detected by flow cytometry in all PCR-positive samples (n=10) and also in 16/23 PCR-negative samples. In PCR-negative samples, neoplastic plasma cell levels were below 0.2% of total leucocytes (median 0.06%), consistent with the limits of sensitivity of the PCR assay in patient samples. The results indicate that flow cytometric analysis is applicable to a greater proportion of patients than IgH-PCR in general, and also has a greater sensitivity for detection of residual neoplastic cells than consensus-primer IgH-PCR.

Comparison of conventional monitoring with flow cytometric analysis

In this group of patients, 22% (10/45) achieved an immunofixation-negative complete remission with the remaining 78% (35/45) achieving a partial response to induction
therapy with C-VAMP. High dose melphalan increased the complete remission rate to 73% (33/45) with 27% (12/45) remaining immunofixation positive. Neoplastic plasma cells were detectable at three months post-transplant in 27% (9/33) of the complete remission patients, and in 92% (11/12) of partial remission patients. In order to determine the reproducibility of the technique, 34 representative samples were re-analysed retrospectively in a blinded fashion. Differences were noted in only 2/34 samples, in one case due to operator error on prospective analysis. In the other case, neoplastic plasma cells were present at a level close to the limit of detection of the flow assay, and the sample was prospectively reported as showing a normal plasma cell profile, but having evidence of residual disease on retrospective analysis. The clinical features suggest that the retrospective analysis was more accurate, as the patient remained immunofixation-positive. However, the results reported in this paper are from prospective analysis to avoid potential bias. This retrospective analysis demonstrates that the assay has good reproducibility, but re-analysis of borderline samples using larger numbers of cells may be beneficial in future studies.

For patients achieving a complete remission post-transplant, the median time to achieve a negative immunofixation was 2.9 months post-transplant, with 12% (4/33) taking over six months to show undetectable levels of paraprotein. To assess whether neoplastic plasma cell levels showed the same kinetics, sequential samples were assessed in a cohort of 12 patients with neoplastic plasma cells detectable at three months post-transplant. A median of 2 further samples were analysed (range 1-7) with a median follow up of 15 months from transplant. In 11/12 patients the levels of neoplastic plasma cells increased steadily, whilst one patient had a stable level of neoplastic plasma cells (i.e. within 0.05% of previous samples) for 17 months, then levels increased until clinical relapse occurred at 38 months. Thus, once neoplastic
plasma cells are detected, the levels increase progressively until overt clinical progression.

This data suggests that analysis of neoplastic plasma cells is more sensitive than immunofixation in the majority of cases, and can be performed at a single time point as once neoplastic cells are detected they do not decrease in level.

**Use of the flow cytometric assay improves prediction of outcome in comparison to standard criteria**

In this series of patients, attainment of an immunofixation-negative complete remission was associated with improved progression-free survival, but this only became apparent at 20 months post-transplant (Figure 2).

*Figure 2: patients attaining a complete remission have prolonged progression-free survival*
Patients with detectable paraprotein by immunofixation had a median progression-free survival of 21.5 months (95% confidence interval 20 – 23), whereas only 12/33 immunofixation-negative patients have progressed with a median 30 month follow up (P=0.002, Log-rank). Overall survival at 5 years was 77% for the complete responders compared to 52% for the partial responders, but this did not reach statistical significance (P=0.16, Log Rank).

When the presence or absence of neoplastic plasma cells was used to define response a similar pattern was seen, although there was slightly better separation between the two arms in the first year post-transplant in comparison to conventional monitoring (Figure 3).

**Figure 3:** Presence of neoplastic plasma cells at three months post-transplant predicts early relapse.
Neoplastic plasma cells were detectable in the bone marrow of 42% of patients (19/45) at three months post autologous transplant. Patients with detectable neoplastic plasma cells had a median progression-free survival of 20 months (95% confidence interval 18 – 23), whereas only 9/26 in the group of patients with no neoplastic plasma cells have progressed with a median 34.5 months follow up (P=0.0034, Log-rank). Survival at 5 years was 64% for the group with neoplastic plasma cells compared to 76% for those with no detectable neoplastic cells, although again this did not reach significance (P=0.28, Log Rank).

Neoplastic plasma cells were detectable in 9/33 (27%) of the IF- patients, and these patients had a median PFS of 20 months. This was significantly poorer than the 24 IF-patients with no detectable neoplastic plasma cells: only 7/24 (29%) have progressed with a median follow-up of 34.5 months (P=0.04, Log rank). There was no difference in overall survival. Immunofixation provided no additional information with respect to progression-free survival for patients with detectable neoplastic plasma cells present. Of 19 patients with neoplastic plasma cells at three months post-transplant, nine became immunofixation-negative and had a median PFS of 20 months, whereas ten remained immunofixation-positive and had a median PFS of 22 months (P=0.6, Log rank).

Univariate and multivariate analysis of outcome was performed for a range of criteria shown in Table 1. The detection of paraprotein by immunofixation was not significant in multivariate analysis, however the detection of neoplastic plasma cells at three months remained significant. This demonstrates the increased sensitivity of the flow cytometric assay for detection of residual disease. In patients with detectable neoplastic plasma cells, approximately half become immunofixation-negative but show an identical outcome to those that remain immunofixation-positive. Presentation
ß2m level was the only other variable to remain significant on multivariate analysis, demonstrating that the presence of residual disease is an independent prognostic factor. Thus the flow assay can discriminate a group of patients who are at risk of relapsing early despite achieving an immunofixation-negative complete remission, independent of presentation ß2m levels.

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Table 1.

Normal plasma cells are present in the bone marrow of most patients at three months post-transplant. If sustained for six months, patients have a significantly better prognosis

At three months post-transplant, normal plasma cells were detectable in 89% (40/45) of patients, and normal CD19+ B-lymphocytes were present in all patients. Previous studies have suggested that the recovery of normal immunoglobulin is a powerful prognostic factor, but the proportion of patients recovering normal levels is much lower. Analysis of sequential samples in 25 patients demonstrated that 16/25 patients had normal plasma cells at second assessment (6-12 months post-transplant), of which all 16 recovered normal immunoglobulin levels. These patients had a much better prognosis: only 2/16 have progressed at 40 and 49 months post-transplant respectively, with a median follow-up of 39 months. However, the median time to recovery of normal immunoglobulin levels was six months, and ranged from 3 to 15
months. Therefore, recovery of normal immunoglobulin levels is not a suitable parameter for identifying patients requiring further up-front therapy. Of the nine patients who had only neoplastic plasma cells at second assessment, all had continued immuneparesis. The latter group had a similarly poor outcome to patients with detectable neoplastic plasma cells at three months, showing a median progression-free survival of 16 months from transplantation (range 7 – 39 months).

The data suggest that it is possible to identify two groups of patients with very different outcomes, based on the detection of normal and neoplastic plasma cells at first and second assessment post-transplantation. In a cohort of thirty-five patients, we defined a high risk group (n=23) as those who have neoplastic plasma cells present at three months post transplant or who have only normal plasma cells present at first assessment and only neoplastic plasma cells at second assessment. The low risk group had only normal phenotype plasma cells present at three months post-transplant, and also had normal plasma cells present at second assessment (n=12). Figure 4 demonstrates a highly significant difference in progression-free survival between these groups (P<0.0001). In addition, there is also a significant difference in overall survival (P=0.036) with a five year survival of 100% for the low risk group, compared to 54% in the high risk group.
Figure 4: normal and neoplastic plasma cell levels post-transplant predict outcome

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Normal plasma cells only at 3 months post-transplant, still present at 6 months post-transplant

Either neoplastic at 3 months, or normal only at 3 months with neoplastic only at 6-12 months
DISCUSSION

In this study, we have assessed the clinical relevance of minimal disease monitoring in patients with multiple myeloma post high dose chemotherapy using conventional criteria, as well as flow cytometric and PCR approaches. Flow cytometric analysis has not been widely used as a method for residual disease analysis in multiple myeloma, but has previously been shown to be extremely effective in chronic lymphocytic leukaemia\(^\text{13}\). It has been demonstrated that a unique neoplastic plasma cell phenotype is identifiable in over 98% of patients, and that the technique can be extremely sensitive\(^\text{15}\). We have demonstrated in this study that a relatively simple flow cytometric technique can identify a group of patients with particularly good prognosis, and a second group with a much poorer progression-free and overall survival.

More recently identified neoplastic markers may allow further improvement of flow cytometric disease monitoring in myeloma\(^\text{16}\). However, during this study we have identified several factors that are essential for residual disease assessment. Factors that affect any residual disease assay are the use of good quality ‘first-pull’ marrow aspirate, and the analysis of sufficient leucocytes (preferably 100,000 – 500,000). Particularly relevant to flow cytometric analysis is the exclusion of B-progenitor cells, which have a similar phenotype to normal plasma cells (CD38\(^++\)CD19\(^+\)CD56\(^-\)). These are best excluded by their lack of CD138 expression.

Numerous PCR strategies have been applied for residual disease monitoring in myeloma, with some showing an improved outcome for those achieving an MRD-negative status\(^\text{8}\), and some showing no difference\(^\text{9}\). A major drawback to PCR analysis is the relatively low number of patients with an amplifiable IgH
rearrangement using consensus primers, as a result of a high degree of somatic hypermutation in the neoplastic cells\textsuperscript{6}. Analysis using consensus primer PCR is relatively insensitive, and provides no additional information to immunofixation\textsuperscript{3}. Patient specific ASO-PCR approaches are more sensitive, but also more labour-intensive and cannot differentiate between myeloma plasma cells and clonally related B cells. This is critical since some investigators have detected B cells clonally related to the myeloma plasma cells post-transplant yet their presence does not predict early relapse, possibly because these cells may not be proliferative\textsuperscript{17-18}. This may explain why most patients remain ASO-PCR-positive post-autologous transplant, and monitoring therefore does not always predict outcome in this setting\textsuperscript{7}.

As in previous studies, we have demonstrated that patients achieving a complete remission have an improved progression-free survival in comparison to partial remission patients\textsuperscript{4}. However, this difference only becomes apparent after prolonged follow-up, and many CR patients show early relapse. Furthermore, it may take up to nine months post-transplant for immunofixation to become negative. Therefore, conventional criteria cannot be used to identify patients who might benefit from additional therapy in the early stages after high dose therapy.

Flow cytometric analysis of normal and neoplastic plasma cells provides a much more powerful prediction of outcome in this cohort, and can be assessed at fixed time points as the result does not depend on variable immunoglobulin half-life. It has been suggested that neoplastic plasma cell levels might remain stable over time in some patients post-transplant\textsuperscript{19}. However, in this study neoplastic plasma cell levels always increased with time until disease progression, and their identification at an early stage predicts a poor outcome. Patients who had only normal plasma cells at first assessment, but only neoplastic at second assessment also had a poor outcome. This
distinct group of patients who respond well initially but relapse quickly have previously been identified in studies of conventional chemotherapy\textsuperscript{20}. Patients with only normal plasma cells at first assessment, and who maintain normal plasma cells at second assessment are nearly all in remission with a median follow-up from transplant of approximately three years. These patients also show recovery of normal immunoglobulin levels that have previously been identified as a good predictor of outcome\textsuperscript{1}. It seems probable that this group of patients will not benefit from additional therapy immediately post-transplant but should be monitored regularly for residual disease on maintenance therapy. In contrast, patients with detectable neoplastic plasma cells at three or six months post-transplant should be considered for further treatment, such as further high-dose therapy, low-intensity conditioning allogeneic transplantation or experimental therapeutic strategies.
REFERENCES


FIGURE LEGENDS

Table 1: Only presentation ß2m levels and detection of neoplastic plasma cells at 3 months post-transplant are significant predictors of outcome in multivariate analysis.

The table shows univariate analysis (Log-rank) of factors that may affect progression-free survival, and multivariate Cox-regression analysis of factors significant in univariate analysis.

Figure 1: flow cytometric detection of neoplastic plasma cells

Figure 1a shows the gating strategy used to detect plasma cells, designed to exclude the majority of contaminating events (particularly B progenitor cells, apoptotic cells and cellular debris) common in post-treatment samples, described in detail in the Methods section. Figure 1b shows representative plots from patients at three months post-transplant. The upper row shows a patient whose bone marrow sample contains only normal phenotype (CD19+CD56dim) plasma cells. The middle row shows a sample with only neoplastic phenotype (CD19- or CD19+56+) plasma cells. The lower row shows a sample containing mostly normal plasma cells with a neoplastic population detectable that represents 15% of total plasma cells.

Figure 2: patients attaining a complete remission have prolonged progression-free survival
Kaplan-Meier analysis of progression-free and overall survival, comparing patients achieving an immunofixation-negative complete remission against those achieving a partial remission only. Survival is shown from time of transplant.

**Figure 3: presence of neoplastic plasma cells at three months post-transplant predicts early relapse**

Kaplan-Meier analysis of progression-free and overall survival, comparing patients with detectable neoplastic plasma cells at three months post-transplant against those with only normal plasma cells present. Survival is shown from time of transplant.

**Figure 4: levels of normal and neoplastic plasma cells immediately post-transplant provide a powerful prediction of both progression-free and overall survival**

Kaplan-Meier analysis of progression-free and overall survival for two groups of patients according to levels of neoplastic and normal plasma cells. Patients who have only normal cells post-transplant and who sustain this recovery have a significantly improved progression-free and overall survival. Those who have neoplastic plasma cells present post-transplant, or who recover normal plasma cells by three months post-transplant but who no normal plasma cells present at 6-12 months, have poor progression-free and overall survival. Survival is shown from time of transplant.
Fow cytometric disease monitoring in multiple myeloma: the relationship between normal and neoplastic plasma cells predicts outcome post-transplantation