Research Doctorate School in Biological and molecular Sciences

Student: Elisa Cannizzo

e-mail: elisacannizzo@libero.it, elisacannizzo12@gmail.com

Supervisor: Prof. Mario Petrini

e-mail: mario.petrini@med.unipi.it

Doctorate Program: Biotechnology for health

Director of the Doctorate Program: Prof. Mario Campa

TITLE OF THE PROJECT

Flow cytometry detection of neoplastic plasma cells: employing a new statistical model in diagnosing and monitoring Multiple Myeloma

Division of Hematology, Department of Clinical and Experimental Medicine, University of Pisa, Second Division of Pathology, Laboratory of Flow Cytometry, AOUP, Pisa, Italy

1. ABSTRACT

In a previous study, we have developed a new statistical diagnostic model that examines what correlation exists between the immunophenotype and clonality detected by FC and histology, defining the diagnostic role of FC in MM. Anyway, although if statistically acceptable, this model was found by analyzing samples collected in just two different laboratories and so, to improve the forecasting efficiency, our next goal will be to perform a multicenter study. Then, according to literature (3), we would like to evaluate the role that this flow cytometric model could have in evaluating response to therapy, in addition to the actual standardized criteria of evaluation. This is a multicenter national and international study. Until now, we have collected data just from laboratory of Lucca, tested the actual model on Lucca's myeloma samples and updated the model including Lucca's samples.

2. INTRODUCTION

Multiple myeloma (MM) is a clonal B-cell disorder in which malignant plasma cells (PCs) accumulate in the bone marrow (BM), producing lytic lesions, excessive amounts of monoclonal protein in the serum or urine and evidence of end-organ damage (hypercalcemia, renal insufficiency, anemia or bone lesions). A conventional diagnosis in MM is based on a variety of laboratory results: morphology, analysis of M component, haematological features, biochemical parameters, immunophenotyping, cytogenetics, DNA ploidy and labelling index-proliferative activity of PC (1). Immunophenotypic studies on MM have now been performed for more than 15 years and flow cytometry (FC) represents an attractive approach, not only for research purposes but also in guiding clinical practice. In this sense, FC has many advantages: **a)** to distinguish among normal, reactive and malignant PC (2-6), **b)** to evaluate the risk of progression from monoclonal gammopathy of unknown significance (MGUS) to MM (7-9), **c)** to detect prognostic markers (5,10-18), **d)** to evaluate minimal residual disease (MRD) (2,19-22) and **e)** to identify new targets for myeloma therapy (5,13,23-39). It may be difficult to define the clonality of a very small phenotypically abnormal plasma cell population in MRD, both by histology and FC. At this time histology remains the gold standard exam but we here define a new diagnostic model that is objective and reproducible at describing the correlation between phenotype and histology. Based upon our experience we

found 8-color FC analysis to be a superior technique for identifying pathologic PC, especially in minimal residual disease (MRD). In a previous study we found that CD19 showed good correlation with the presence of disease using a cut-off of 61% to distinguish between normal and neoplastic PCs. However, this study evaluated the role of each antigen separately in detecting the presence of disease (40) (this study was performed in Massachusetts General Hospital (MGH), Boston. We studied 15 control specimens and 55 patients). In another study (41) (this study was performed in Pisa, at Hematology section, Santa Chiara Hospital, together with the collaboration of MGH. 15 control samples and 177 patients were studied. Patients were from both Santa Chiara Hospital and MGH) we demonstrated that the contribution of different antigens assessed simultaneously improved the correlation with histological results. We described a statistical model where, among all antigens tested, assessing CD19 and CD27 together resulted in the best concordance with histology. Its practical application is simple, rapid and does not require specialized technicians. We propose to use this formula as routine diagnostic tool. It could be used by a simple excel sheet or by a database, where, putting CD19 and CD27 expression values for each patient studied, a value of probability of disease can be obtained. A model plot could also be used for a quick test (41, Figure 1). A difference value of 0.2 could be used as cutoff of concordance between histology and model (41, Table 1). Anyway, although if statistically acceptable, this model was found by analyzing samples collected in just two different laboratories and so, to improve the forecasting efficiency, our next goal will be to perform a multicenter study. Then we would like to evaluate the role that this flow cytometric model could have in evaluating response to therapy, in addition to the actual standardized criteria of evaluation. Thus, a multicenter national and international study, involving 21 Italian laboratories and laboratory of Patholgy of MGH, in Boston, is in progress. Until now, we have collected and analyzed MM samples from Hospital of Lucca.

3. MATERIALS AND METHODS

Control and Patient Samples

All specimens for this study were obtained from Massachusetts General Hospital (MGH) in Boston, the Santa Chiara Hospital (SCH) in Pisa, Italy and Campo di Marte Hospital (CMH) in Lucca, Italy. Control specimens consisted of 15 bone marrow samples obtained from

patients seen by the orthopaedic service of MGH for elective hip replacement. These patients had neither history of plasma cell myeloma (PCM), MGUS nor other hematological diseases. The PCs identified in all these samples were polyclonal and co-expressed CD19+CD56-. Bone marrow samples from 177 patients suspected or known to have plasma cell neoplasms submitted to MGH and SCH laboratories for routine analysis from April 2008 to April 2010 were evaluated by FC, histology and immunohistochemistry. We evaluated patients who were positive for a new diagnosis of MGUS or MM and patients in evaluation post-chemotherapy, post autologous and allogenic stem cell transplant. The 15 control specimens and 105 of the 177 patient specimens were used both to define the statistical model and, after obtaining bone marrow histology results, to test it's applicability retrospectively (Table 2). The remaining 72 patient samples were then used to test the statistical model prospectively (before obtaining bone marrow histology results) (Table 3). Response to therapy was defined according to Durie et al (42, Table 4). The study was approved by the Institutional Review Board of Partners Healthcare and SCH. Then, 64 bone marrow samples from patients suspected or known to have plasma cell neoplasms submitted to the Laboratory of the Hospital of Lucca for routine analysis from September 2010 to October 2012 were evaluated by FC and morphology evaluation. These samples were from male or female Patients, of more than 18 year-old, suspected or known to have plasma cell neoplasms: patients at diagnosis, during follow up, in evaluation after chemotherapy, after autologous bone marrow transplant, after allogenic bone marrow transplant

Peripheral blood samples were submmitted for the following exams: IgG, IgA and IgM quantification, seric and urine Electrophoresis, seric and urine M component, seric and urine free light chains, Beta-2 microglobulin, Serum albumin, Calcium, Haemoglobin, LDH. All data collected are:

1. % of PC by histology
2. % of PC by morphology
3. Soft tissue plasmocitoma (if present)
4. IgG, IgA and IgM quantification
5. Seric and urine Elecrophoresis
6. Seric and urine M component
7. Seric and urine free light chains

8. Beta-2 microglobulin
9. Serum albumin
10. Calcium
11. Creatinine
12. Haemoglobin
13. LDH
14. % of PC by FC
15. % of expression of each marker on plasma cells (CD138, CD38 and CD45 must be collected as MFI)
16. Time of observation (at diagnosis, in follow-up, after chemotherapy, after autologous bone marrow transplant, after allogenic bone marrow transplant).
17. ISS disease stage
18. Hosteolysis
19. Type of diagnosis

Flow cytometry

Bone marrow aspirate specimens were run on a 3-laser FACSCanto (BD Biosciences, San Jose CA) running DiVa 6.1.1 software; instrument calibration was performed and tested weekly. Weekly calibration using CS&T beads was performed as well as compensation using appropriately stained normal peripheral blood samples, as previously described (40,43). Whole bone marrow and hip marrow samples collected in EDTA anticoagulant were washed three times in phosphate-buffered saline (PBS) and resuspended with 1% bovine serum albumin in PBS. 100 µl of washed cells were incubated with each of the following titered monoclonal antibodies and corresponding volumes for 15 minutes at room temperature in the dark: 7 µl CD138-PeCy7(BD Biosciences), 5 µl CD81-allophycocyanin (APC) (BD Biosciences), 3 µl CD200-phycoerythrin (PE) (BD Pharmingen), 3 µl CD221-PE (BD Pharmingen), 3 µl CD45 Amcyan (BD Biosciences), 8 µl CD38-fluorescein isothiocyanate (FITC) (BD Biosciences), 3 µl CD28-PE(BD Biosciences), 8 µl CD19-peridin chlorophyll protein (PerCP) (BD Biosciences), 5 µl CD27-APC, 5 µl CD117-APC (BD Biosciences), 5 µl CD38-APC (BD Biosciences), 7 µl CD33-PacBlue (BD Biosciences), 7 µl CD20-APC-Cy7

(BD Biosciences), 7 µl CD56-PacBlue(BD Biosciences), 7 µl CD10-PacBlue (BD Biosciences), 10 µl of each immunoglobulin κ and λ light chains (DAKO, Carpinteria, CA) PE and FITC respectively. These markers were identified among many other possible choices because they are highly cited and used in detecting PCs and for distinguishing between normal and abnormal PCs (2,38,39). The antibodies were combined as tabulated in Table 5.
Erythrocytes were lysed by incubating with 2 mL of 1:9 diluted BD FACS lysing solution for 10 minutes (10 ml of BD FACS lysing solution in 90 ml of H2O). After incubation, cells were pelleted by centrifugation (416g for 5 min at room temperature), the supernatant was aspirated and the cells washed once in PBS. After the final wash step, cells were resuspended in 0.5 ml of 1% of paraformaldehyde in PBS. To detect cytoplasmic κ and λ light chain expression, the Fix and Perm (Caltag, Burlingame CA) cytoplasmic staining kit was used as follows; after incubating the specimen with anti-surface antibodies for 15 minutes, the sample was washed with 1X PBS and added to 100 µl of Fix and Perm reagent A solution. After 15 minutes of incubation the cells were washed again and added to 100 µl of Fix and Perm reagent B solution together with anti- κ and λ light chain antibodies and incubated for 15 minutes. The supernatant was discarded and cells re-suspended into 0.5 ml of PBS. In order to obtain a minimum of 100 PCs in the analyzed region, the number of total bone marrow cells acquired for each sample tube ranged from 160,000 to 1,000,000 (2, 40, 41). In order to detect MRD at 0.01%, the minimum number of total collected events required was approximately 106. To optimally detect PCs, we analyzed a combination of CD138, CD38 and CD45 together with light scatter, as previously described (2, 40, 41). Briefly, an initial immunofluorescence analysis gate was devised using CD38 versus CD138 expression, a second one using CD38 brightly positive versus CD45 positive and negative cells and the last one using light scatter characteristics (high FSC and low SSC). These three intersecting gates were utilized to obtain a defined PC population in multidimensional space. Co-mingling gated lymphocytes served as internal controls in both patient and control specimens for studying the associated PC population; thus the expression of antigens detected on PCs was compared directly to that of autologous gated lymphocytes in the patient and control specimen. The expression of all 17 antigens detected on PCs was then analyzed by a logistic regression statistic to estimate the probability that the event termed “presence of disease” occurred, in comparison with histology.

Histologic and Immunohistochemical Examination

Tissue examination of patient samples was performed to determine the presence of monoclonal or polyclonal PCs and disease status. Two-micron thick B+-fixed, paraffin-embedded bone marrow core biopsy sections were prepared, de-paraffinized and rehydrated according to standard laboratory protocols. A combination of ethylenediaminetetraacetic acid and boric acid in Tris buffer (CC1 reagent, Ventana) was used for antigen retrieval before primary antibody incubation. Immunohistochemistry for CD138 (Serotec, Raleigh, NC; 1/30 dilution) was performed using avidin-biotin peroxidase complex with a peroxidase-labeled detection system on a Benchmark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ) using validated staining protocols. In situ hybridization for kappa and lambda immunoglobulin light chains was performed using HX Automatic System Benchmark (Ventana) according to the manufacturer's instructions. Signals were detected using the Alkaline Phosphatase Enhanced Detection Kit (Ventana).

Histology defined presence or absence of disease on the basis of determining PC clonality.

Cases with plasma cell neoplasms were confirmed to have a clonal PC population as determined by one of the following: 1) >10% morphologically abnormal PCs on the bone marrow aspirate and fulfilling WHO criteria for a diagnosis of PCM or 2) A monotypic kappa or lambda positive PC population identified in the bone marrow biopsy sample by CD138 immunohistochemistry and in situ hybridization for kappa and lambda light chains.

Statistical analysis

A logistic regression statistic was used to estimate the probability that the event termed "presence of disease" occurred. An independent variable (antigen) was defined as statistically significant when the *p*-value of it's corresponding coefficient found was ≤ 0.05 by the likelihood ratio test. To determine whether or not the logistic regression model fit, the Chi-squared goodness-of-fit test was used and the model was accepted for a *p*-values ≥ 0.05. The logistic regression analysis have been done by a stepwise method, by which all variables are

initially tested and then, just those resulted statistically correlated were included in the model. Analyses were performed with the software STATGRAPHICS XV.

4. RESULTS

In a previous study we found that CD19 showed good correlation with the presence of disease using a cut-off of 61% to distinguish between normal and neoplastic PCs. However, this study evaluated the role of each antigen separately in detecting the presence of disease (40) (this study was performed in Massachusetts General Hospital (MGH), Boston. We studied 15 control specimens and 55 patients). In another study (41) (this study was performed in Pisa, at Ematology section, Santa Chiara Hospital, together with the collaboration of MGH. 15 control samples and 177 patients were studied 120 to found the model and the remaining to test it prospectively). Patients were from both Santa Chiara Hospital and MGH) we demonstrated that the contribution of different antigens assessed simultaneously improved the correlation with histological results. We described a new diagnostic statistical model, which is objective and reproducible, to observe the correlation between immunophenotype and histology. This model defines the effective diagnostic role FC could have in MM and above all in detecting MRD.

Of the 17 unique antigens tested in this study, CD19 (p-value=0.0000) and CD27 (p-value=0.0001) we found to be statistically correlated with histological results distinguishing between the absence or presence of disease and thus deemed eligible to be included in the model. Table 6 shows each of these antigens model values obtained for the coefficient, p-value, standard error, odds ratio and 95% confidence intervals. We can see that CD19 and CD27 have negative coefficients which indicates that if the values of CD19 and CD27 are high, the probability that the event "presence of disease" occurs, is low. These findings are in accord with existing literature (2, 39, 44, 45).

The parameter b_0 (constant) shows the cut off point where antigens' values change from high to low probability and vice versa. It also defines a baseline probability of disease when the variables CD19 and CD27 are both zero as follows:

$$\text{Baseline probability} = \exp(-b_0) / (1 + \exp(-b_0))$$

The probability *P* that the event "presence of disease" occurs ranges between 0 and 1 where 0 means that the event does not occur (absence of disease), while 1 means that the event occurs (presence of disease). This model makes predictions about the probability that the event "presence of disease" will happen for each patient, given the values of the variables CD19 and CD27 (Figures 1 and 2). The value obtained via the model ranging between 0 and 1 was compared with what was observed by histological results (0 or 1). Histology defined presence or absence of disease on the basis of determining PC clonality and is the gold standard in detecting the presence of disease in MM. The Chi-Squared Goodness of Fit Test was used to determine whether or not this logistic regression model fit; the test measures the overall difference between what it was expected to see via the model versus what it was observed by histological results (R^2-adjusted). With an R^2 adjusted of 70%, we showed a correlation exists between FC analysis and histological results. Therefore, even if not perfect, this model is certainly acceptable and potentially might be improved by increasing the sample size. A multicenter study was so organized (centers from Italy and Boston) and it is in progress. Until now, we have collected 64 MM samples from Lucca Hospital. We have tested the actual statistical model on these cases and we found that it works in 95% of cases. A difference value of 0.2 could be used as cutoff of concordance between histology and model (41, Table 1). We then recalculated the statistical model, including samples from Lucca to those one from Pisa and Boston, and we found that CD19 (*p*-value=0.0000) and CD27 (*p*-value=0.0008) are still the two antigens best correlated with histological results, with an R^2 adjusted of 73,6% (Tables 7), so similar to that one previousely found (41). On all samples studied (120 from Pisa and Boston and 64 from Lucca), the percentage of concordant cases was 91.3%, with 4.7% discordant cases and 4% uncertain cases. Discordant cases were CD19 and CD27 positive myeloma cases and those cases where a monoclonal plasma cell population was detected together with a polyclonal plasma cell population by flow cytometry. Cases having uncertain results by the model have a difference between the value of the event observed by histology and the value of the event predicted by the model ranging between 0.20 and 0.46. These values are less than 0.50 and so could be considered acceptable, although, to make the

model very restrictive, a value of difference between 0 and 0.20 was considered acceptable. On 64 samples from Lucca we also tested if any correlation exist between markers and all other laboratoristic and clinical data collected and it was not found at moment. We do not exclude that probably collecting more samples, we could find a better correlation with these other parameters..

5. DISCUSSION

The role of FC is important in detecting MRD in myeloma when there are fewer than 5% of PCs by morphology and a very small percentage of PCs to detect clonality by histology. Unlike morphology and histology, FC is not dependable in providing quantitative information about MM, but does provide qualitative information for assessing both the immunophenotype and light chain clonality of PCs. The percentage of MRD detection in FC (97%) is comparable to that obtained by histology (96%) and better then those obtained by conventional molecular analysis (60-70%) and in situ hybridization (50%). Immunofixation electrophoresis is not informative about response until six months after the end of therapy, due to the time required for the M component to disappear (22). No single antigen has been reported to systematically differentiate abnormal PCs from their normal counterparts and it is not possible to distinguish between normal and abnormal PCs on the basis of CD19 and/or CD56 alone, due to the presence of clonal CD19+ and/or CD56- PCs and polyclonal CD19- and or CD56+ PCs (2,13,45). It is important to detect clonality and immunophenotype together, especially when attempting to detect MRD. In that situation, it is difficult to evaluate the κ/λ ratio, because the restriction for one light chain is evident when the clonal population represents at least the 30% of the polyclonal background. For this reason some investigators look for the cytoplasmic heavy chain during the follow-up post-therapy (2). It has been reported that MRD detection is clearly associated with the total PC percentage and that for a low percentage of PC, it is not possible to accurately distinguish between truly MRD-negative or patients with very low levels of residual disease (46). CD19, CD56, cytoκ and cytoλ are known to be the antigens most involved in distinguishing between normal and abnormal PCs but in this investigation, testing many different antigens together, it was

indicated which of them was significantly correlated with histological results (and with the presence or absence of disease) through a logistic regression test.). In another study (41) (this study was performed in Pisa, at Hematology section, Santa Chiara Hospital, together with the collaboration of MGH. 15 control samples and 177 patients were studied, 120 to found the model and the remaining to test it prospectively). We demonstrated that the contribution of different antigens assessed simultaneously improved the correlation with histological results. We described a new diagnostic statistical model, which is objective and reproducible, to observe the correlation between immunophenotype and histology. This model defines the effective diagnostic role FC could have in MM and above all in detecting MRD. With an R^2 adjusted of 70%, we showed a correlation exists between FC analysis and histological results. Among all the antigens that were tested, CD19 (*p*-value=0.0000) and CD27 (*p*-value=0.0001) were found to be statistically correlated with histologic results and useful in distinguishing between the absence and presence of disease and thus eligible to be included in the model (Table 6, Figures 1 and 2). Therefore, even if not perfect, this model is certainly acceptable and potentially might be improved by increasing the sample size. A multicenter study was so organized (centers from Italy and Boston) and it is in progress. In the last year we have collected 64 MM samples from Lucca Hospital. We have tested the actual statistical model on these cases and we found that it works in 95% of cases. We then recalculated the statistical model, including samples from Lucca to those one from Pisa and Boston, and we found that CD19 (*p*-value=0.0000) and CD27 (*p*-value=0.0008) are still the two antigens best correlated with histological results, with an an R^2 adjusted of 73,6% (Table 7), so similar to that one previousely found. On all samples studied (120 from Pisa and Boston and 64 from Lucca), the percentage of concordant cases was 91.3%, with 4.7% discordant cases and 4% uncertain cases. Discordant cases were CD19 and CD27 positive myleoma cases and those cases where a monoclonal plasma cell population was detected together with a polyclonal plasma cell population by flow cytometry. Cases having uncertain results by the model have a difference between the value of the event observed by histology and the value of the event predicted by the model ranging between 0.20 and 0.46. These values are less than 0.50 and so could be considered acceptable, although, to make the model very restrictive, a value of difference between 0 and 0.20 was considered acceptable. This model makes predictions about the probability that the event “presence of disease” will occur for each patient, given the values of

the variables CD19 and CD27. Interestingly, cytoK, cytoλ and CD56 expressions were not correlated with histological results. CD56 expression was reported in 70-80% of MM patients (47,48). In previous studies we found that CD27 played a similar role as CD56 in detecting the presence of disease and so could be considered an additional helpful antigen in this regard (40, 41). As we described above, it is not always possible to evaluate K/λ ratio in MRD when there is a low percentage of plasma cells; the statistical model found in this study seems to overcome this problem by using the values associated with CD19 and CD27. CD19 is an antigen gained in early stage of B differentiation and always present in the majority of normal PCs. On the other hand, only a small percentage (<5%) of patients with MM express this antigen (13). Mateo et al showed that MM patients with CD19 positive cells have a less favourable outcome then those lacking CD19 expression, with a progression free survival (PGS) and an overall survival (OS) approximately one or two years shorter. CD27 is expressed on B cell subsets and on the majority of peripheral T cells (39, 49). It has been reported that the expression of CD27 on the PCs of MGUS patients is similar to that on normal PCs. The loss of CD27 in MGUS has been linked to MM progression. The lack of CD27 expression is usually coupled to the loss of CD19. Conversely, the loss of CD19 is not always associated with that of CD27 in monoclonal gammopathies (44). Gene expression profile studies also reveal that CD27 expression is reduced in myeloma cells compared to the elevated expression in normal PCs (44). This model offers a fast way to detect the presence of disease by flow cytometry, which started with a small panel of antigens consisting of CD38, CD138, CD45, CD19 and CD27. We cannot exclude that more and/or different antigens could be involved in this correlation in other samples. In my last published paper, we also evaluated how the results obtained by the model are concordant with the actual classification of response to chemotherapy (41, Table 4). We observed that on 62 treated patients, 35 were positive either by histology or model and classified 10 as in PR and 25 as in NR. 16 were instead negative on both, and all classified as in CR (Table 8). Discordant cases between histology and model were 19 and CD27 positive myeloma cases (three 1/0 cases in Table 9) and those cases where a monoclonal plasma cell population was detected together with a polyclonal plasma cell population by flow cytometry (two 0/1 cases in Table 9).. In these last cases our model was shown to be more accurate than histology. We feel this model may be

adopted as an additional method to evaluate disease response to chemotherapy, considering the limits of immunofixation and histology in detecting MRD.

6. CONCLUSIONS

In agreement with previously reported results, our diagnostic model confirmed the importance of the absence of CD19 expression in detecting the presence of MM. In contrast, CD56 seemed not to have a significant role. We also found that the absence of CD27 expression was an additional marker correlated with the presence of disease. By application of this model we found an objective and reproducible way to assess the malignancy of plasma cells which was highly correlated with histological results and permits the reproducible detection of MRD. Its practical application is simple, rapid and does not require specialized technicians. Our results are similar to those reported in our previous paper (41).

7. FUTURE PERSPECTIVE

We propose to use the formula we found as routine diagnostic. Anyway, although if statistically acceptable, this model was found by samples collected in three different laboratories and so, to improve the forecasting efficiency, we are organizing a multicenter study by which we would like to rich the aims:

Primary:

1. to test and update the model found in *Cannizzo et al, Am J Clin Pathol 2012* (3) on a larger scale, *i.e.* on more samples and from more laboratories. In this way, we will try to improve the forecasting efficiency of the model.
2. to confirm that cytometric detection of CD19 and CD27 is predictive of the presence/absence of disease (as assessed by other criteria, e.g. immuno-histology, serum and/or urine M component, evidence of endorgan damage, serum and urine free light chains).

Secondary:

3. to asses a new model in distinguishing between MGUS and MM.
4. to evaluate how this flow cytometric model works in evaluating MRD after therapy, in particular at time +100 after HDT/ASCT (2).
5. to assess the prognostic role of surface markers defined by flow cytometry
6. to develop a biologic scoring system to better define the prognosis of MM.

8. References

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FIGURE AND TABLES:

Table 1

Value of the estimated P that the event occurs	*Interpretation*
0.2	The probability is 20% that the event is *abnormal* and it is unlikely to indicate the presence of disease.
0.8	The probability is 80% that the event is *abnormal,* and it is very likely to indicate the presence of disease.
0.5	The probability is 50% that the event is *abnormal*, and it is maximally uncertain at predicting the presence or absence of disease

Table 2

Diagnosis	Time	Response to chemotherapy
94 MM	27 at diagnosis	NA
	3 post-allo-transplantation	1 PR, 2 CR
	26 post-auto-transplantation	11 CR, 15 PR
	38 post-chemotherapy	12 CR, 16 PR, 5 SD, 5 PD
11 MGUS	4 at diagnosis and 7 in follow-up	NA

Table 3

Diagnosis	Time	Response to chemotherapy
62 MM	45 post-chemio	12 CR, 6 PR, 16 SD, 11 PD
	17 post-auto	10 CR, 6 PR
	1 post-allo	1 CR
10 MGUS	2 at diagnosis and 8 in follow-up	NA

Table 4

Response subcategory	Response criteria
Complete response (CR)	Negative immunofixation of serum and urine and Disappearance of any soft tissue plasmacytomas, and <5% plasma cells in bone marrow
Stringent complete response (sCR)	CR as defined above plus Normal FLC ratio and Absence of clonal cells in bone marrow by immunohistochemistry or immunofluorescence
Very good partial response (VGPR)	Serum and urine M-component detectable by immunofixation but not on electrophoresis or ≥90% or greater reduction in serum M-component plus urine M-component o100mg per 24 h
Partial response (PR)	≥50% reduction of serum M protein and reduction in 24-h urinary M protein by ≥90% or to <200mg per 24 h If the serum and urine M protein are unmeasurable, a ≥50% decrease in the difference between involved and uninvolved FLC levels is required in place of the M protein criteria If serum and urine M protein are unmeasurable, and serum free light assay is also unmeasurable, ≥50% reduction in bone marrow plasma cells is required in place of M protein, provided baseline percentage was ≥30% In addition to the above criteria, if present at baseline, ≥50% reduction in the size of soft tissue plasmacytomas is also required
Stable disease (SD)	Not meeting criteria for CR, VGPR, PR or progressive disease

Progressive disease (PD)	Increase of 25% from lowest response value in any one or more of the following: Serum M-component (absolute increase must be ≥0.5 g/100 ml)c and/or Urine M-component (absolute increase must be ≥200mg per 24 h) and/or Only in patients without measurable serum and urine M-protein levels: the difference between involved and uninvolved FLC levels (absolute increase must be 4100 mg/l) Bone marrow plasma cell percentage (absolute % must be ≥10%) Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas Development of hypercalcemia (corrected serum calcium 411.5 mg/100 ml) that can be attributed solely to the plasma cell proliferative disorder

Table 5

AmCyan	*FITC*	*PE*	*PerCP*	*APC*	*PeCy7*	*PacificBlue*	*APCCy7*
CD45	**CD38**	**CD221**	**CD19**	**CD27**	**CD138**	**CD56**	
CD45	**CD38**	**CD200**	**CD19**	**CD81**	**CD138**	**CD10**	
CD45	**CD38**	**CD28**	**CD19**	**CD117**	**CD138**	**CD33**	
CD45	**cyto λ**	**cyto κ**	**CD19**	**CD38**	**CD138**	**CD56**	**CD20**

Table 6

Predictor	*Coefficient*	*P-Value*	*Standard Error*	*Odds Ratio*	*95% confidence intervals*	
					Lower	*Upper*
Constant	11,5199		2,93897		5,69943	17,3404
CD19 %	-0,0843	0,0000	0,0194	0,9191	-0,1228	-0,0458
CD27 %	-0,0741	0,0001	0,0256	0,9285	-0,1250	-0,0232

Table 7

Parameter	*Estimate*	*Standard Error*	*Estimated Odds Ratio*	*P-Value*	*95% confidence intervals*	
					Lower Limit	*Upper Limit*
CONSTANT	**9,33505**	**2,02225**			**5,34469**	**13,3254**

CD19 %	-0,0838652	0,0168476	0,919555	0,0000	-0,117109	-0,0506211
CD27 %	-0,0466216	0,0159689	0,954449	0,0008	-0,078132	-0,0151111

Table 8

Response to Chemotherapy	[6]H/M (0=absence of disease; 1=presence of disease; U=uncertain)					
	0/0	*1/1*	*0/1*	*1/0*	*0/U*	*1/U*
[1]CR	16	0	2	0	5	0
[2]PR	0	10	0	2	0	0
[3] SD	0	15	0	0	0	1
[4]PD	0	10	0	1	0	0
total	16	35	2	3	5	1

Table 9

Cases	*H/M*	*n*
Discordant	1/0	5
	0/1	8
Uncertain	0/U	9
	1/U	2

Figure 1

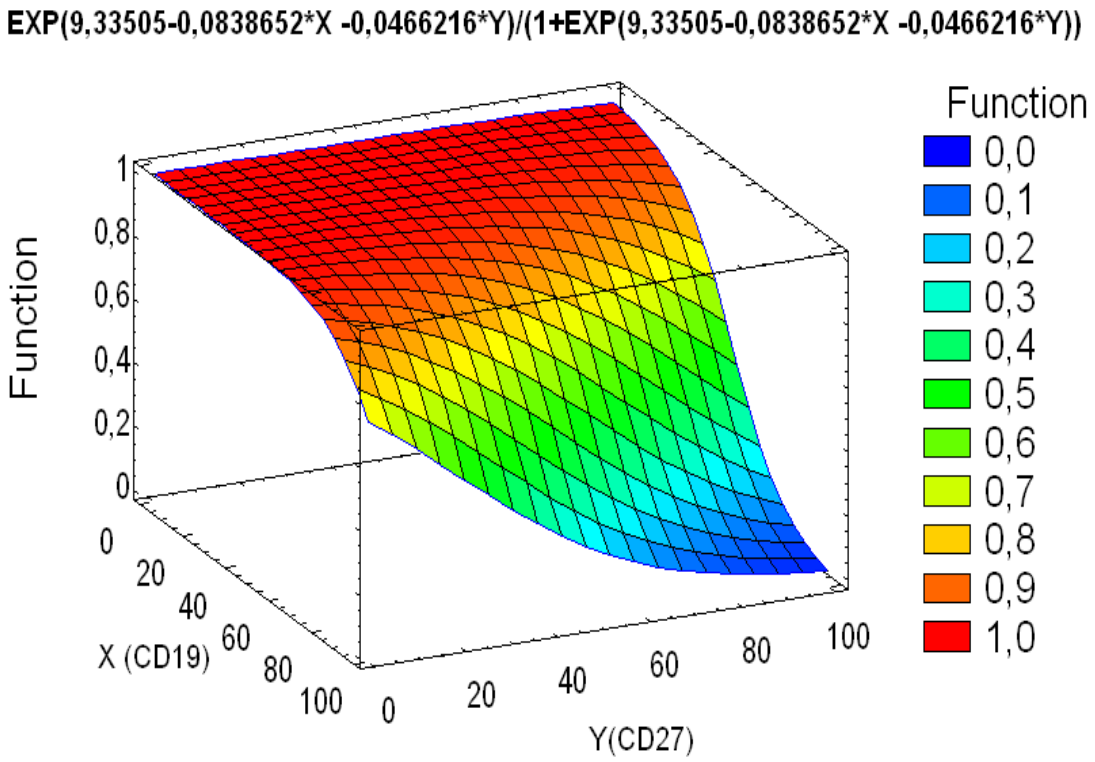
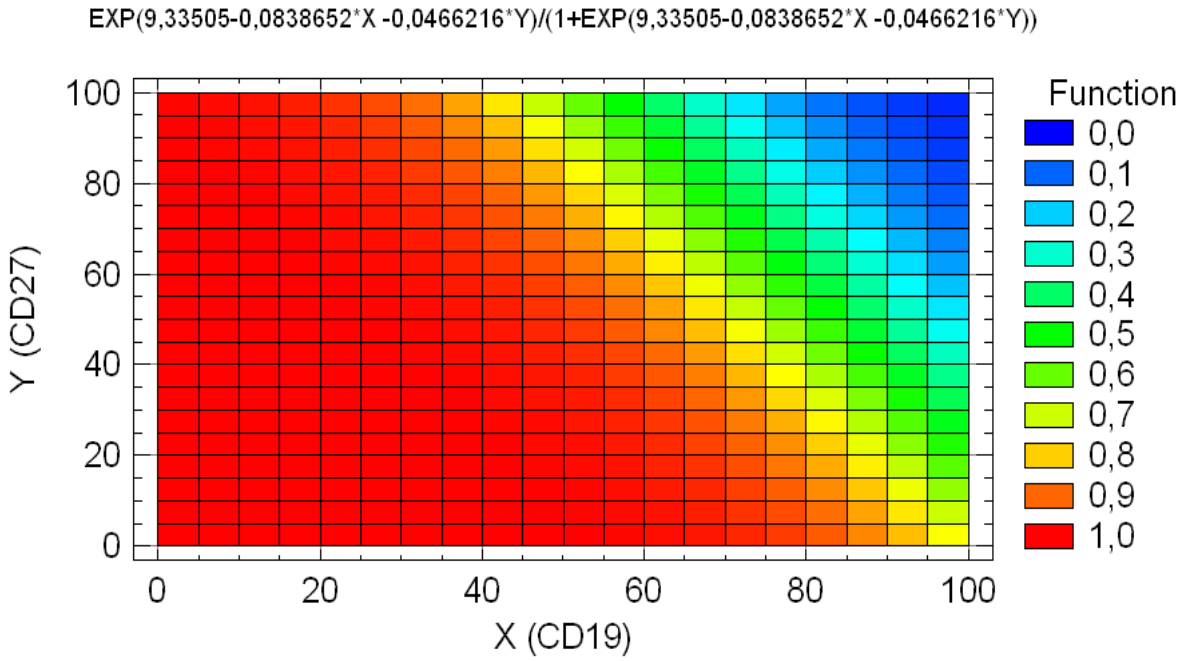

EXP(9,33505-0,0838652*X -0,0466216*Y)/(1+EXP(9,33505-0,0838652*X -0,0466216*Y))
Function
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
100
X (CD19)
0
20
40
60
80
100
Y(CD27)
Function
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0

Figure 2



$$P = \frac{\exp(9{,}33505 - 0{,}0838652*\text{CD19 \%} - 0{,}0466216*\text{CD27 \%})}{1+\exp(9{,}33505 - 0{,}0838652*\text{CD19 \%} - 0{,}0466216*\text{CD27 \%})}$$