

Serum calcium is an independent prognostic factor of overall survival in Mexican patients with multiple myeloma

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ABSTRACT

Objective. To evaluate the impact of different prognostic factors that has been suggested to be useful in predicting the survival of patients with multiple myeloma (MM). **Materials and methods.** A longitudinal prospective study was conducted on 24 adult Mexican patients diagnosed with primary MM. The levels of expression of CD38, CD138 and cyclin D1 were analyzed in plasma cells (PCs) from patients and mononuclear cells from healthy donors. Serum levels of lactate dehydrogenase, creatinine, calcium, β_2 microglobulin and interleukin-6 (IL-6) as well as hemoglobin and platelet count were taken into consideration. **Results.** CD138 and cyclin D1 levels in absolute numbers were significantly overexpressed in malignant PCs. A positive correlation was noted between cyclin D1 and CD38 expression levels in malignant PCs. IL-6 and serum calcium were also positively correlated in MM patients. Cyclin D1 overexpression was not associated with better overall survival (OS). Normal calcium levels were associated with better overall survival (OS). Serum calcium was the only variable correlating with better OS in Cox regression analysis. **Conclusion.** Serum calcium is an independent prognostic factor of OS in a population of Mexican patients with MM.

Key words. CD138. Cyclin D1. Prognostic factors. Multiple myeloma. Calcium.

El calcio sérico es un factor pronóstico independiente para la supervivencia global en pacientes mexicanos con mieloma múltiple

RESUMEN

Objetivo. Evaluar el impacto de diferentes factores pronóstico, descritos como útiles para predecir la supervivencia de los pacientes con mieloma múltiple. **Material y métodos.** Se realizó un estudio prospectivo longitudinal en 24 pacientes adultos con diagnóstico de mieloma múltiple (MM) primario. Se analizaron los niveles de expresión de CD38, CD138 y ciclina D1 en las células plasmáticas de los pacientes y en las células mononucleares de donadores sanos (control) por citometría de flujo. También se consideraron los niveles de lactato deshidrogenasa, creatinina, calcio, β_2 microglobulina e interleucina 6 (IL-6) en suero; así como la cifra de hemoglobina y la cuenta de plaquetas. **Resultados.** Los niveles de CD138 y ciclina D1 en valor absoluto, se sobreexpresaron significativamente en las células plasmáticas malignas. Se observó una correlación positiva significativa entre los niveles de expresión de ciclina D1 y CD38 en las células plasmáticas malignas. También se obtuvo una correlación positiva significativa entre los niveles de IL-6 y el calcio sérico en los pacientes con MM. La sobreexpresión de ciclina D1 no se asoció con una mejor supervivencia global. Los niveles de calcio normal se asociaron con una mejor supervivencia global. El nivel de calcio sérico fue la única variable que se asoció con una mejor supervivencia en el análisis de regresión de Cox. **Conclusión.** El nivel de calcio en suero mostró ser un factor pronóstico independiente para la supervivencia global en la población estudiada de pacientes mexicanos con MM.

Palabras clave. CD138. Ciclina D1. Factores pronóstico. Mieloma múltiple. Calcio.

INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy characterized by clonal proliferation of plasma cells (PCs) in the bone marrow and, usually, the presence of a monoclonal Ig in the serum and/or urine. At diagnosis, MM patients have signs and symptoms such as anemia, bone lesions, hypercalcemia, or renal dysfunction. Different blood, bone marrow, urine and imaging studies are necessary in order to diagnose, stage and monitor this disease.

Durie and Salmon included monoclonal protein level and type, hemoglobin, serum calcium, serum creatinine and extent of bone lesions as prognostic factors for this disease.¹ Later, in the 1980s, serum β_2 microglobulin (β_2 -M) emerged as the most powerful prognostic factor—a simple and reliable predictor of survival duration.^{2,3} In the 1990s, interleukin-6 (IL-6) emerged as a prognostic factor in MM.⁴ Subsequently, other prognostic factors were introduced, including serum C-reactive protein,⁵ albumin, and the proliferative activity of PCs in the bone marrow determined by flow cytometry analysis of the S-phase of the cell cycle.⁶ In addition, the karyotype obtained from conventional cytogenetics has emerged as another important prognostic factor in patients with myeloma.⁷ In the karyotype, deletion of chromosome 13 (del 13) is the most common significant prognostic factor found.^{8,9}

On the other hand, in the last few years plasma cell disorders have been shown to be associated with recurrent chromosomal abnormalities: almost half of MM patients have nonrandom translocations involving the Ig heavy chain (IgH) locus on 14q32 as well as one of five well-defined chromosome pairs: 11q13 (cyclin D1), 6p21 (cyclin D3), 4p16 (fibroblast growth factor receptor-3 and multiple myeloma SET domain), 16q23 (c-maf) and 20q11 (mafB).¹⁰ All IgH locus translocations except t(11;14) are unfavorable prognostic factors.¹¹ In contrast, t(11;14)(q13;q32) is the most common of these with a frequency of 14-59% as determined by conventional cytogenetics or fluorescence in situ hybridization (FISH).^{12,13} The latter translocation results in upregulation of the proto-oncogene CCND1 which codes for the cell cycle regulatory protein cyclin D1. Recently, cyclin D1 overexpression has been determined to be a favorable prognostic variable in newly diagnosed MM patients treated with high-dose chemotherapy and single or double autologous transplantation.¹⁴

On the other hand, although most of the previously mentioned laboratory variables have been shown to be prognostic factors in univariate analy-

sis,¹⁵ it is important to remember that most of these factors are interrelated. Furthermore, most of them have been examined in different population groups, but which of them is the most powerful prognostic factor in a Mexican population group remains unknown.

OBJECTIVE

To evaluate the impact of different prognostic factors such as platelet count, hemoglobin, serum creatinine, serum calcium, lactate dehydrogenase (LDH), serum β_2 -M and cyclin D1 levels on long-term survival in MM patients.

MATERIAL AND METHODS

Patients

The present longitudinal prospective study was conducted from August 2005 to January 2009 on patients from the Department of Hematology at La Raza Medical Center. After obtaining informed consent, bone marrow and blood samples were taken from 24 previously untreated symptomatic patients with MM and blood was taken from 16 healthy donors. Diagnosis was established through clinical history, physical examination, X-ray images and common blood and urine laboratory tests, and morphological diagnosis by Wright's stain, acid phosphatase testing, and immunophenotyping of bone marrow samples. The main clinicobiological characteristics of the patients are shown in table 1.

Blood count

Blood samples taken from patients before treatment were collected in BD Vacutainer® tubes with EDTA (K2 EDTA 7.2 mg, REF 368171). The blood count was obtained with an automated cell counter (Coulter Immunotech, Marseille, France). Platelet count and hemoglobin levels were taken into consideration in the statistical analysis.

Blood chemistry and immunoassays

Blood samples taken from patients before treatment were collected in serum tubes (BD Vacutainer® SST TM, REF 368159). Blood chemistry was determined using a clinical chemistry analyzer (ARCHITECT c8000, Abbott, IL, USA). Serum levels of creatinine, calcium and LDH were taken into consideration in

Table 1. Clinicobiological characteristics of patients with multiple myeloma.

Characteristic	%
• Number of patients: 24	
• Sex	
Male	67
Female	33
• Immunoglobulin subtype	
IgG	53
IgA	19
Bence Jones protein	14
Non-secretory	14
• Median age (years)	59
Range	38-74
• Median serum calcium (mg/dL)	9.5
Range	7.2-15
• Median hemoglobin (g/dL)	10.25
Range	6.3-16.8
• Median platelet count (10 ⁹ /mL)	258
Range	69.9-521
• Median creatinine (mg/dL)	1.13
Range	0.5-5.1
• Median LDH (U/L)	218
Range	80-500
• Median b ₂ Microglobulin (mg/L)	7.27
Range	1.47-19.22
• Median IL-6 (pg/mL)	39.7
Range	26.4-195.6

IgG: immunoglobulin G. IgA: immunoglobulin A.

the statistical analysis. Two enzyme-linked immunosorbent assays (ELISA) were performed per manufacturer instructions to determine serum IL-6 and β_2 -M levels (Human IL-6 Quantikine® ELISA Kit catD6050 and Human β_2 -M cat DBM200, R&D Systems®, Minneapolis MI, USA, respectively). Serum IL-6 and β_2 -M levels were obtained using an Emax precision microplate reader and SoftMax Pro 4.3 LS software (Molecular Devices, USA).

Flow cytometry analysis

Bone marrow samples were taken from patients before treatment. Similarly, blood was taken from

healthy donors. Immediately after collection, the samples were processed in a single laboratory by the same research person. The total number of nucleated cells was obtained from the blood count. Cell number and the plasma cell population analyzed were obtained by flow cytometry using FACSCalibur equipment (Becton Dickinson, San Jose, CA, USA). For staining, whole blood aliquots containing up to 10⁶ leukocytes were incubated with adequate amounts of each monoclonal antibody for 15 min at room temperature. Red blood cell lysis was performed using FACS Lysing Solution (Becton Dickinson). Cells were washed twice with phosphate buffered saline before acquisition was made.

To determine cyclin D1, cells were treated with a permeabilizing solution (Becton Dickinson) according to manufacturer instructions. CD38, CD138 and cyclin D1-expressing PCs were determined using the following combination of antibodies: cyclin D1-FITC/CD38-PE/CD138-APC. Adequate isotype controls included IgG-FITC, IgG1-PE and IgG-APC (Becton Dickinson). CellQuest Pro software (Becton Dickinson) was used for data acquisition. The parameters acquired per cell were forward scatter (FSC) and side scatter (SSC). Four fluorescence events were acquired per analysis and stored in list-mode files. A minimum of 100,000 cells was acquired. To determine cyclin D1-expressing PCs in patients, we gated the total CD38 cell population (R1) on the CD38-PE vs. cyclin D1-FITC dot plot and then selected the PCs (R2) on the SSC-H vs. FSC-H dot plot, as shown in figure 1A, 1B. PCs expressing and not expressing cyclin D1 were identified as cyclinD1+CD38+ and/or CD138+, and cyclinD1-CD38+ and/or CD138+ respectively, as shown in figure 1C, 1D. Absolute cell numbers for each marker were obtained by multiplying the fraction of each marker within the fraction gate value by the fraction of PCs and by the absolute number of white blood cells per microliter as determined by automated cell counting.

The lymphocyte-monocyte rich region R1 in healthy donors was selected on the SSC-H vs. FSC-H dot plot (all events). Absolute cell numbers for each marker were derived by multiplying the fraction of each marker within the fraction gate value by the fraction of mononuclear cells (MNcells) and by the absolute number of white blood cells per microliter as determined by automated cell counting.

Statistical analysis

Data were analyzed using PASW Statistics 18 v18.0.0 (IBM, Somers, NY, USA). Comparisons bet-

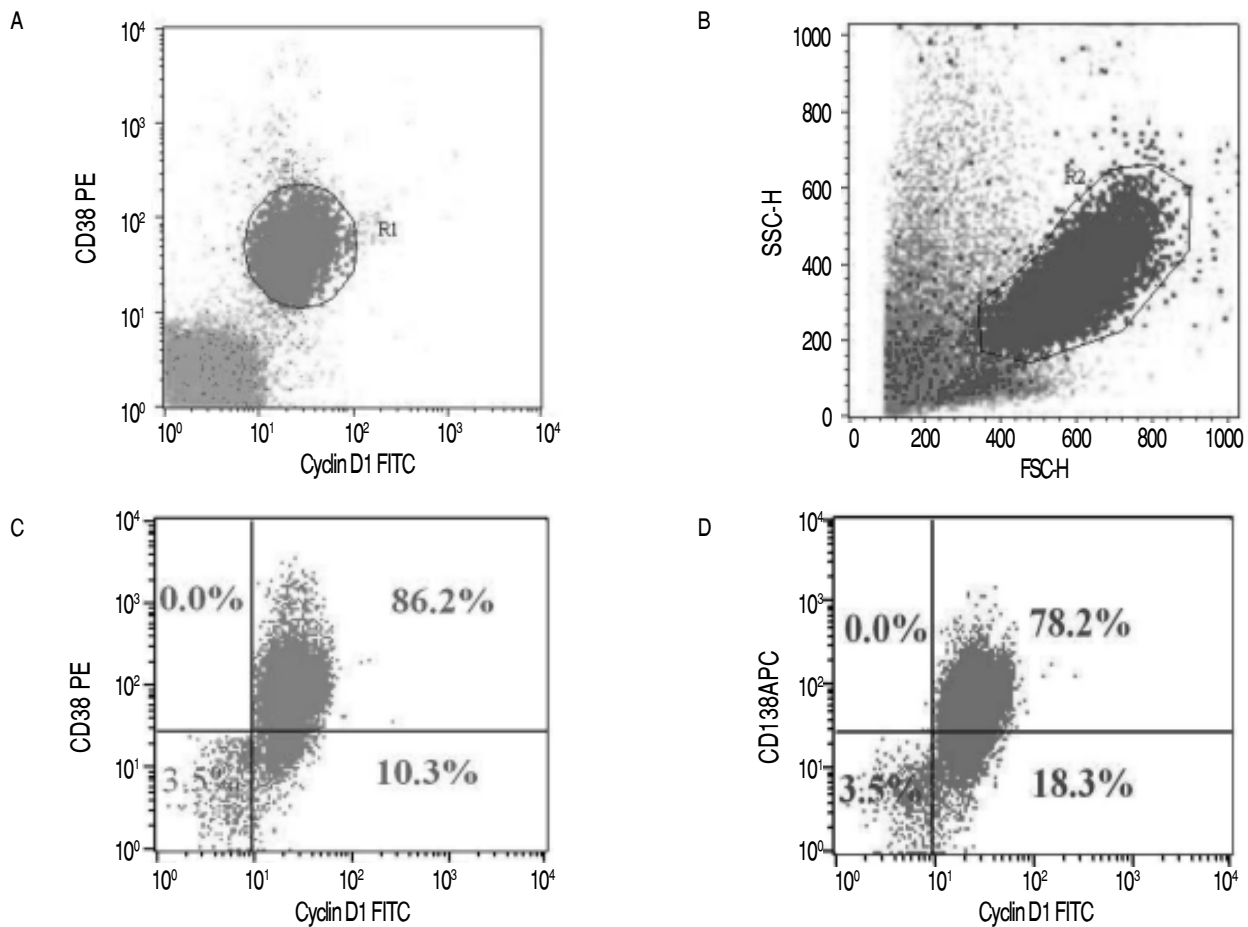


Figure 1. Data analysis in MM patients. A. Selection of the CD38+ region (R1) on the CD38-PE vs. cyclin D1-FITC dot plot. B. Selection of the CD38+ region (R2) from R1 on the SSC-H vs. FSC-H dot plot. C. Percent expression of cyclin D1+ and CD38+ in malignant PCs. D. Percent expression of cyclin D1+ and CD138+ in malignant PCs.

ween patient and healthy donor values were performed with the Mann-Whitney U test for non-parametric variables. Spearman correlation was used to find the association between cyclin D1 expression and immunophenotypic marker expression, platelet count, β_2 -M, LDH, hemoglobin, serum creatinine and serum calcium, as well as to correlate IL-6 with serum calcium and β_2 -M. To dichotomize patients by their levels of expression of cyclin D1, protein overexpression was said to exist when expression was > 30% since in all healthy donors percent expression was below this value. Survival curves were generated by the Kaplan-Meier method. Differences between two curves were analyzed with the log-rank test. Overall survival (OS) was defined as the time from diagnosis to death (regardless of cause of death) or to last-documented contact with the patient. A Cox proportional hazards model was used to evaluate the independent effect of variables such as pla-

telet count, serum calcium, serum creatinine, LDH, hemoglobin, β_2 -M and cyclin D1 on OS.

The level of significance was set at $p < 0.05$ for all analyses.

RESULTS

Blood count, blood chemistry and ELISA

Table 1 shows the median and range of values of the different variables in MM patients at diagnosis.

Expression of cyclin D1, CD38 and CD138 in plasma cells

Figure 2 shows a dispersion graph of points representing the expression values of each marker in absolute cell numbers in patients and in healthy donors.

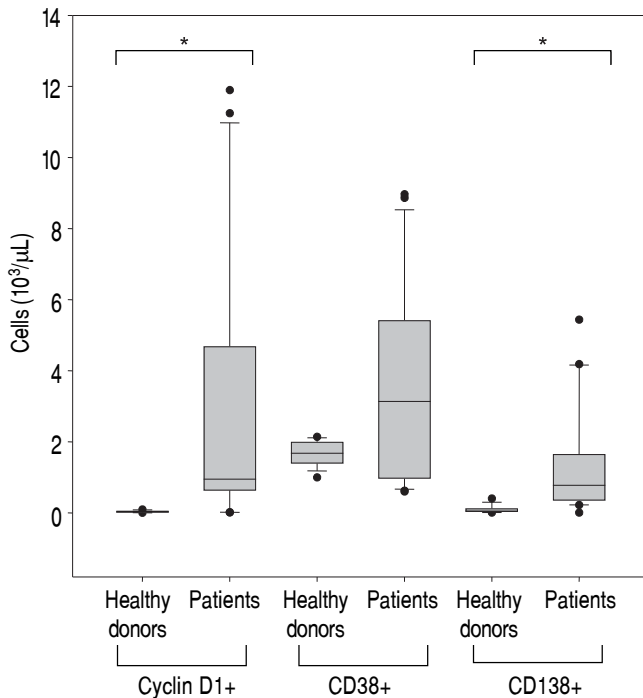


Figure 2. Absolute expression of immunophenotypic markers in MM patients and healthy donors. Shown are median, 75th percentile (box), standard deviation (whiskers) and outliers (dots), * $p < 0.05$, Mann-Whitney U-test.

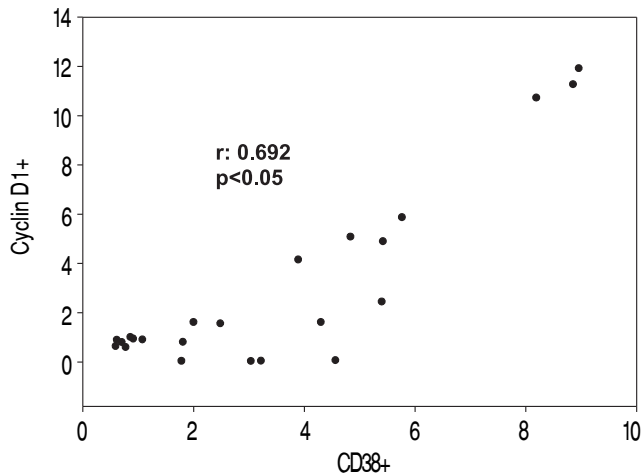


Figure 3. Correlation between cyclin D1 and CD38 expression in MM patients. A positive correlation was found between the total expression levels of cyclin D1 and CD38 in absolute numbers. (Cell $\times 10^3/mL$).

Expression levels of cyclin D1, CD38 and CD138 were higher in patient PCs than in healthy donor MNcells. Significant differences were found between patients and healthy donors in the expression levels of cyclin D1 and CD138.

Correlation between the total expression of cyclin D1 and IL-6 and other markers in MM patients

A positive correlation was found between the total expression levels of cyclin D1 and CD38 ($r = 0.692$) in absolute numbers, as shown in figure 3. No correlation was noted between the total expression levels of cyclin D1 and CD138 in absolute numbers or the total expression levels of cyclin D1 and platelet count, β_2 -M, LDH, hemoglobin, serum creatinine and serum calcium. On the other hand, a positive correlation was found between IL-6 and serum calcium ($r = 0.677$, data not shown). IL-6 and β_2 -M showed no correlation.

Survival curves

Median time to follow-up after diagnosis was 17.5 months (range, 1-54 months).

Median survival time for all patients was 43 months.

Patients presenting normal serum calcium had better OS than those with hypercalcemia (median 49 vs. 22 months respectively, significantly different values) as shown in figure 4A. Patients who overexpressed cyclin D1 had a median OS of 43 months and those who did not had a median OS of 22 months. No significant differences were found between these two patient groups, as shown in figure 4B.

Hypercalcemia was the only variable correlated with poorer OS, i.e. calcemia was the significant independent variable for survival, while serum creatinine, LDH, platelet count, hemoglobin, β_2 -M and cyclin D1 proved not to be independent variables, as shown in table 2.

Table 2. Risk analysis of the different variables evaluated in MM patients.

Variable	HR	95% CI	*p value
Serum calcium	2.1	0.014 - 0.91	< 0.02
Platelet count	1.7	0.006 - 4.37	0.1
β_2 -microglobulin	1.4	0.03 - 1.75	0.1
Hemoglobin	1.1	0.62 - 14.7	0.1
Creatinine	1.3	0.028 - 2.57	0.2
Cyclin D1	0.8	0.052 - 3.53	0.4
LDH	0.9	0.060 - 2.99	0.9

HR: hazard ratio. CI: confidence interval. *Cox proportional hazards model.

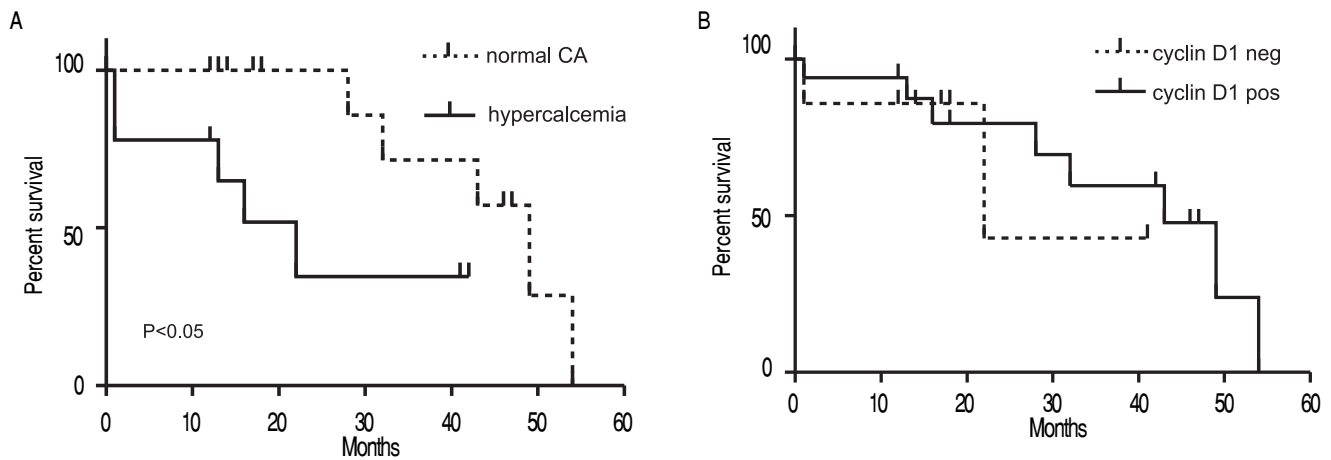


Figure 4. Comparison of Kaplan-Meier curves of overall survival (OS) in MM patients, analyzed with the log-rank test. A. To find whether serum calcium affects OS, patients were divided based on the presence of normal serum calcium or hypercalcemia at diagnosis; OS curves were significantly different ($p < 0.05$). B. To find whether cyclin D1 expression affects OS, patients were divided based on presence/absence of cyclin D1 overexpression at diagnosis; no significant differences were found.

DISCUSSION

In recent years additional prognostic factors have been proposed to predict survival in MM patients. However, despite significant efforts and advances, some of these factors remain difficult to determine in all patients. In the present study we evaluated the role of different prognostic factors such as platelet count, hemoglobin, serum creatinine, serum calcium, LDH, β_2 -M and cyclin D1 which have been suggested by previous studies to be useful in predicting MM patient survival. To this end, we carried out flow cytometry analysis in order to determine cyclin D1 in malignant PCs of MM patients. In addition, we conducted long-term follow-up on our patients, which allowed us to identify which of these prognostic factors were better predictors of survival. Serum calcium was found to be the best predictor of long-term survival in MM patients.

To characterize PCs, we determined the simultaneous expression of CD38 and CD138 on the surface of these cells, since both markers have been suggested to be the best combination for identifying and discriminating malignant PCs in other cell populations in hematological samples.¹⁶⁻¹⁷

The phenotype of cells in our study revealed that CD38+ MNcells from healthy donors and malignant PCs from patients expressed CD38 and CD138, and percent expression was similar to that reported by Bataille, *et al.*¹⁷ They stated that CD138 is overexpressed and CD38 is underexpressed in myeloma cells as compared to normal PCs. However, they do

not provide expression values for these markers in absolute cell numbers, but as intensity of expression. We estimated and present results as absolute expression values. Our results show that these phenotypic markers are overexpressed in PCs of MM patients. We noted significant overexpression of CD138. Also, we determined expression of cyclin D1 in PCs expressing CD38 and CD138. This conferred a major advantage to flow cytometry over other methods such as immunohistochemistry, FISH and RT-PCR. Results show significant overexpression of cyclin D1 in malignant PCs from MM patients. Moreover, cyclin D1 levels in PCs of patients were more than 30-fold higher than the median value in CD38+ MNcells from healthy donors.

An interesting finding in our study was the positive correlation between cyclin D1 and CD38 expression values in MM patients. This overexpression of cyclin D1 in malignant PCs with high levels of expression of CD38 is explained by the fact that cyclin D1 is a cell cycle protein that is present in proliferating cells. These results corroborate previous descriptions about cyclin D1 overexpression in immature myeloma cells¹⁸ with high levels of expression of CD38.¹⁹

On the other hand, we found that better survival was associated with normal serum calcium but not with cyclin D1 overexpression. Results show that 37.5% of our MM patients presented hypercalcemia at diagnosis. This is higher than the frequency reported in other population groups.¹⁵ However, a frequency of 25.45% has been previously reported in a

Mexican population²⁰ and our sample size was smaller than those reported earlier. OS was significantly longer in patients with normal serum calcium than in those with hypercalcemia. When patients were sorted by serum calcium level and their clinical data were checked, we found that all patients with hypercalcemia presented stage III at diagnosis (data not shown). On the other hand, since patients are diagnosed in advanced stages of the disease, with high tumor burden and high secretion of IL-6, and elevated serum IL-6 levels have been linked to increased myeloma cell proliferation and disease severity,^{4,21} this is associated with poor OS. Our results show a positive correlation between IL-6 and serum calcium in MM patients, and are consistent with reports by De La Mata, *et al.*,²² who showed that IL-6 enhances hypercalcemia and bone resorption *in vivo*. One hypothesis that may help explain these findings is that bone disease was not too advanced in patients presenting normal serum calcium. These patients had a greater possibility of controlling disease progression and thus better OS. In contrast, patients with hypercalcemia are reported to present most frequently the highest tumor mass, and destructive osteolytic bone disease²³ supported by the vicious cycle between bone destruction and myeloma expansion.²⁴ Cyclin D1 was overexpressed in 70% of patients in the present study. During long-term follow-up of our patients, results show that OS was longer in patients overexpressing cyclin D1 than in those who did not, although no statistically significant difference was noted. Small sample size and a low frequency (30%) of patients who did not express cyclin D1 may explain why no significant differences were found between OS curves in our study. We were therefore unable to associate cyclin D1 overexpression with better survival.

On the other hand, no correlation was found between cyclin D1 and known prognostic factors such as β_2 -M, LDH, platelet count, hemoglobin, serum creatinine and serum calcium. These results are consistent with reports by Soverini, *et al.*, and Rasmussen, *et al.*,^{14,25} who found no correlation between cyclin D1 and known prognostic factors.

Unlike other studies,^{2,3,5} β_2 -M levels proved not to be a useful prognostic predictor of OS in our population. This may be due to the small size of our sample. Increasing the sample size may result in more precise conclusions.

Finally, the present study shows that normal serum calcium was the most important independent prognostic factor at diagnosis associated with longer overall survival in a Mexican population group.

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