

Quantification of cyclin D1 and D2 proteins in multiple myeloma identifies different expression patterns from those revealed by gene expression profiling

Ignacio J. Cardona-Benavides,^{1,2} Irena Misiewicz-Krzeminska,³ Elizabeta A. Rojas,^{1,2} Cristina De Ramón,^{1,2} Antonio Sanz-Solas,^{1,2} Isabel Isidro,^{1,2} Dalia Quwaider,^{1,2} Aida M. López-Guerrero,^{1,2} Myriam Cuadrado,^{1,2} María-José Calasanz,^{4,5} Laura Rosiñol,⁶ Joaquín Martínez-López,^{5,7,8} Jesús F. San Miguel,^{4,5} María-Victoria Mateos,^{1,2,5} Luis A. Corchete^{1,2,5#} and Norma C. Gutiérrez^{1,2,5#} on behalf of the GEM/PETHEMA (Grupo Español de Mieloma/Programa para el Estudio de la Terapéutica en Hemopatías Malignas) cooperative study group

¹Hematology Department, University Hospital of Salamanca, Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain; ²Cancer Research Center-IBMCC (USAL-CSIC), Salamanca, Spain; ³Department of Experimental Hematology, Institute of Hematology and Transfusion Medicine, Warsaw, Poland; ⁴Clínica Universidad de Navarra, Centro de Investigaciones Biomédicas Aplicadas (CIMA), Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain; ⁵Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Spain. ⁶Hospital Clinic of Barcelona, Instituto de Investigaciones Biomédicas August Pi I Sunyer (IDIBAPS), Barcelona, Spain; ⁷Spanish National Cancer Research Center (CNIO), Madrid, Spain and ⁸Hematology Department, Hospital 12 de Octubre, Medicine Department, Complutense University Madrid, Madrid, Spain

#LAC and NCG contributed equally as senior authors.

Correspondence: N.C. Gutiérrez
normagu@usal.es

Received: May 5, 2023.

Accepted: August 18, 2023.

Early view: August 31, 2023.

<https://doi.org/10.3324/haematol.2023.283445>

©2024 Ferrata Storti Foundation

Published under a CC BY-NC license



Abstract

Upregulation of a cyclin D gene determined by expression microarrays is an almost universal event in multiple myeloma (MM), but this finding has not been properly confirmed at the protein level. For this reason, we carried out a quantitative analysis of cyclin D proteins using a capillary electrophoresis nanoimmunoassay in newly diagnosed MM patients. Exclusive expression of cyclin D1 and D2 proteins was detected in 54 of 165 (33%) and 30 of 165 (18%) of the MM patients, respectively. Of note, cyclin D1 or D2 proteins were undetectable in 41% of the samples. High levels of cyclin D1 protein were strongly associated with the presence of t(11;14) or 11q gains. Cyclin D2 protein was detected in all the cases bearing t(14;16), but in only 24% of patients with t(4;14). The presence of cyclin D2 was associated with shorter overall survival (hazard ratio =2.14; $P=0.017$), although patients expressing cyclin D2 protein, but without 1q gains, had a favorable prognosis. In conclusion, although one of the cyclins D is overexpressed at the mRNA level in almost all MM patients, in approximately half of the patients this does not translate into detectable protein. This suggests that cyclins D could not play an oncogenic role in a proportion of patients with MM (*clinicaltrials.gov.identifier: NCT01916252*).

Introduction

Dysregulation of D-type cyclins has been invoked as an early and unifying oncogenic event in multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS), which is a premalignant condition.¹⁻³ Gene expression profiling (GEP) has demonstrated that 98% of patients with MM overexpressed *CCND* mRNA: *CCND1*, *CCND2* and *CCND3* were overexpressed in about 46%, 41% and 3% of patients, respectively; additionally, *CCND1* and

CCND2 were co-expressed in 8% of patients. *CCND1* and *CCND2* expression was found to be mutually exclusive in almost all cases.¹⁻³ Thereafter, these results seem to have been confirmed when MM samples were analyzed by RNA sequencing.⁴

CCND1 mRNA overexpression is attributable in 15-20% of cases to t(11;14), which leads to high levels of cyclin D1. In most of the other MM patients with *CCND1* overexpression, polysomy of chromosome 11 is the probable cause of this *CCND1* dysregulation.^{5,6} On the other hand, *CCND2* overex-

pression apparently arises from mechanisms that are not directly associated with *CCND2* gene abnormalities, but rather are a consequence of the dysregulation of other genes. *MAF* and *MAFB* leucine zipper transcription factors, which are involved in the t(14;16) and t(14;20) translocations, respectively, have been shown to upregulate *CCND2* through their transactivation function, leading to an increase in the rate of cell division and DNA synthesis.⁷ Beyond the *MAF* family, *ZKSCAN3*, a zinc finger transcription factor, has been described as inducing *CCND2* promoter activity and thereby cyclin D2 upregulation.⁸ More recently, our group described that the shortening of *CCND2* 3'UTR by alternative polyadenylation with the consequent loss of miRNA binding sites is also involved in *CCND2* upregulation.⁹ Dysregulation of D-type cyclins and their associated pathways is common in both solid and hematological malignancies.¹⁰ A central role of D-type cyclins is the regulation of cyclin-dependent kinases (CDK), in particular CDK4 and CDK6, to promote cell-cycle progression (G1-S transition) through the phosphorylation and inactivation of the RB tumor-suppressor protein.¹¹⁻¹⁴ The oncogenic role of cyclin D1 is well established in many tumors, and its amplification and overexpression is generally associated with negative outcomes.¹⁵⁻¹⁷ However, the overexpression of *CCND1*, which is strongly associated with t(11;14) and trisomy 11, does not confer an unfavorable prognosis on patients with MM.^{3,18} The function of cyclins D2 and D3 in tumorigenesis has been less thoroughly explored, and their consequences for survival are sometimes mixed¹⁹. Particularly in MM, overexpression of *CCND2* has been associated with poor prognosis, probably due to the predominance of high-risk cytogenetic alterations in this group of patients.²⁰⁻²⁴

The overexpression of cyclin D mRNA in almost all MM cases contrasts with the generally low proliferation rate observed in tumor plasma cells.^{1,3,25} One possible explanation for this is that cyclins D may perform other functions that are unrelated to cell-cycle progression.¹³ Another possibility is that protein levels of cyclins D may not be high or stable enough to trigger cell-cycle transition from G1 to S phase. In this regard, few studies have analyzed the expression of cyclin D proteins, and most of those that have done so used immunohistochemical techniques.^{26,27} Although immunohistochemistry (IHC) provides valuable information about the expression of proteins in the tissue context, the technique is usually semiquantitative and uses arbitrary cutoff levels. The adoption of capillary electrophoresis nanoimmunoassay (CNIA) technology may help overcome these drawbacks, given its capacity to facilitate the quantitative analysis of proteins with high sensitivity and its requirement for only nanogram amounts of sample.^{28,29} In order to shed light on these elusive aspects related to the expression of cyclins D in MM, we carried out a quantitative analysis of cyclin D proteins in a large cohort of newly diagnosed MM (NDMM) patients who were homogeneously

treated according to GEM2012 clinical trial. We compared the results with *CCND1* and *CCND2* mRNA levels quantified by quantitative real-time polymerase chain reaction (qRT-PCR). The impact of cyclin D expression on survival of MM patients was also explored.

Methods

Patient samples

A total of 165 samples from NDMM patients treated as part of the Spanish Myeloma Group clinical trial GEM2012 (*clinicaltrial.gov*. Identifier: NCT01916252) were included in the study,³⁰ which was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. Informed consent was required prior to patient participation in the clinical trial. Patients were treated with six cycles of VRD (bortezomib, lenalidomide and dexamethasone) as induction followed by autologous stem cell transplantation with melphalan 200 *versus* busulfan-melphalan, and consolidation treatment with two cycles of VRD. CD138⁺ plasma cells were isolated from bone marrow aspirates using the AutoMACS immunomagnetic separation system (Miltenyi-Biotec, Germany). Plasma cell purity was > 80% in all the cases. All samples were immediately frozen in RLT⁺ buffer (Qiagen, Germany) and stored at -80°C for further analysis, as previously described.²⁹ RNA, DNA and protein were extracted using an AllPrep DNA/RNA Mini Kit (Qiagen). Proteins were extracted by ice-cold acetone precipitation.^{28,29} Cytogenetic analysis by fluorescence *in situ* hybridization (FISH) for detecting *IGH* translocations, 17p deletions, 1q gains and 1p losses was carried out in all patients, as previously described.

The main characteristics of patients are summarized in the *Online Supplementary Table S1*. This cohort of patients was representative of the whole GEM2012 trial dataset.³⁰

Capillary electrophoresis nanoimmunoassay

CNIA was performed using the WES machine (ProteinSimple, California, EEUU) according to the manufacturer's protocols, and as previously used by our group.^{28,29,31}

Primary antibodies used in the study under optimized conditions were: rabbit monoclonal cyclin D1 (Abcam [Cambridge, UK], ab134175, dilution 1/50), rabbit monoclonal cyclin D2 (Cell Signaling [Danvers, EEUU], #3741, dilution 1/50) and rabbit monoclonal GAPDH (Cell Signaling, #2118, dilution 1/50). Cyclin D1 and D2 protein peaks were normalized with respect to the GAPDH median area under the peak. Expression of each protein was represented relative to that of GAPDH. A more extensive protocol for relative protein quantification by CNIA has been reported elsewhere.^{28,29}

Quantitative real-time polymerase chain reaction

RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer. Approximately 200 ng of total RNA

were reverse-transcribed into cDNA using the SuperScript II First-Strand Synthesis kit (Thermo Fisher, California, EEUU). Gene expression of *CCND1* and *CCND2* were evaluated with TaqMan qRT-PCR assays, Hs00765553_m1 and Hs00153380_m1, respectively (Thermo Fisher). The *PGK1* gene (Hs00943178_g1, Thermo Fisher) was used as the endogenous control. Relative expression was calculated whereby $\Delta Ct = Ct \text{ housekeeping gene} - Ct \text{ target gene}$.

Statistical analysis

Continuous variables were assessed for normality using the Shapiro-Wilk test. Differences between the experimental groups were analyzed using two-tailed *t* tests or Mann-Whitney U tests, as appropriate, for normally and non-normally distributed continuous variables, respectively. The mclust package (v.5.4.10) was used to model these data as a Gaussian mixture in which the optimal number of components would be determined from the Bayesian Information Criterion (BIC) values of the adjusted models. Fisher's exact test was used to evaluate the association between the resulting categorical variables. The Spearman rank test was used to estimate correlations. Survival curves were depicted using the Kaplan-Meier estimator and were compared with the log-rank test in the survival R package (v.3.3-1). The endpoints included in this survival analysis were time to progression (TTP) and overall survival (OS). The events of interest for TTP were restricted to disease progression and relapse, whereas OS was defined as the time from diagnosis until the date of death from any cause. Values of $P < 0.05$ were considered statistically significant for all tests. Statistical analyses were carried out in R (v.4.2.1).

Results

Expression profile of cyclin D1 and cyclin D2 proteins

Expression of cyclin D1 and cyclin D2 proteins was highly variable among the samples, particularly in the case of cyclin D1. Expression values of cyclin D1 ranged from 0 to 15.05, while those of cyclin D2 varied from 0 to 1.18 (Figure 1A). Excluding non-expressed values, the non-parametric coefficients of variation for cyclin D1 and cyclin D2 were 96% and 70%, respectively.

Patients were divided into four groups based on their cyclin D1 and cyclin D2 protein expression: expression of cyclin D1 exclusively (54/165, 33%); expression of cyclin D2 exclusively (30/165, 18%); co-expression of both proteins (14/165, 8%); no expression of either cyclin D protein (67/165, 41%) (Figure 1B).

The group of MM patients expressing only cyclin D1 contained all the 23 cases with t(11;14) and 55% (21/38) of the cases with 11q13 gains. In other words, cyclin D1 expression was associated with t(11;14) or 11q13 gains in 82% (44/54) of the patients. We next dichotomized the expression of this group of patients by fitting a Gaussian mixture model that

differentiated two groups, one with a high level of cyclin D1 expression (cyclin D1 > 0.057), and the other with a low level (cyclin D1 ≤ 0.057) (Figure 1C). Eighteen of the 23 patients (78%) with t(11;14) were classified in the group with high cyclin D1 expression, while only two of the 38 patients (5%) with 11q13 gains were included in that group (Figure 1D). *IGH* translocations other than t(11;14) were rarely found in the group of patients expressing only cyclin D1. In fact, t(4;14) was detected in only two cases that also featured 11q13 gain, which were in turn classified into the low cyclin D1 expression group.

In the group of patients who exclusively expressed cyclin D2, none had t(11;14) as expected, although 11q13 gain was present in three of the 30 patients (10%). The distribution of the other cytogenetic abnormalities in this group was as follows: t(4;14) and t(14;16) were each present in 13% (4/30) of the cases; 1q gains, and 1p and 17p deletions, were found in 80% (24/30), 27% (8/30) and 20% (6/30) of cases, respectively. t(14;16), 1q gains and 1p deletions were significantly enriched in the group of patients expressing only cyclin D2 compared with the other MM patients (13% vs. 0%, $P < 0.001$; 80% vs. 40%, $P < 0.001$; 27% vs. 10%, $P = 0.03$, respectively). FISH studies yielded normal results in only two of the 30 patients expressing solely cyclin D2. In the same way as for cyclin D1, patients with cyclin D2 expression were dichotomized into two groups, one with high cyclin D2 expression (cyclin D2 > 0.058) and the other with low expression (cyclin D2 ≤ 0.058) (Online Supplementary Figure S1A). Cytogenetic abnormalities were uniformly distributed throughout the two groups (Online Supplementary Figure S1B).

In the group of patients co-expressing both cyclins D a preference for the expression of one of them was observed in 10 of the 14 patients (71%) (Figure 1E). Based on the level of expression of each cyclin D, most cases (71%) expressed low levels of cyclin D1 and D2. Two cases each exhibited high levels of expression of cyclin D1 and of cyclin D2. None of the patients belonging to this group showed t(11;14), while 11q13 gain was detected in three patients who expressed low levels of both cyclins D.

Finally, the largest group of patients analyzed (41%) expressed neither of the cyclins D. The distribution of cytogenetic abnormalities analyzed by FISH within this group is summarized and compared with the other three groups of cyclin D expression in the Online Supplementary Table S2. Interestingly, more than half of the patients with t(4;14) did not express cyclin D2, whereas all the four samples with t(14;16) did express it.

Expression profiles of *CCND1* and *CCND2* mRNA

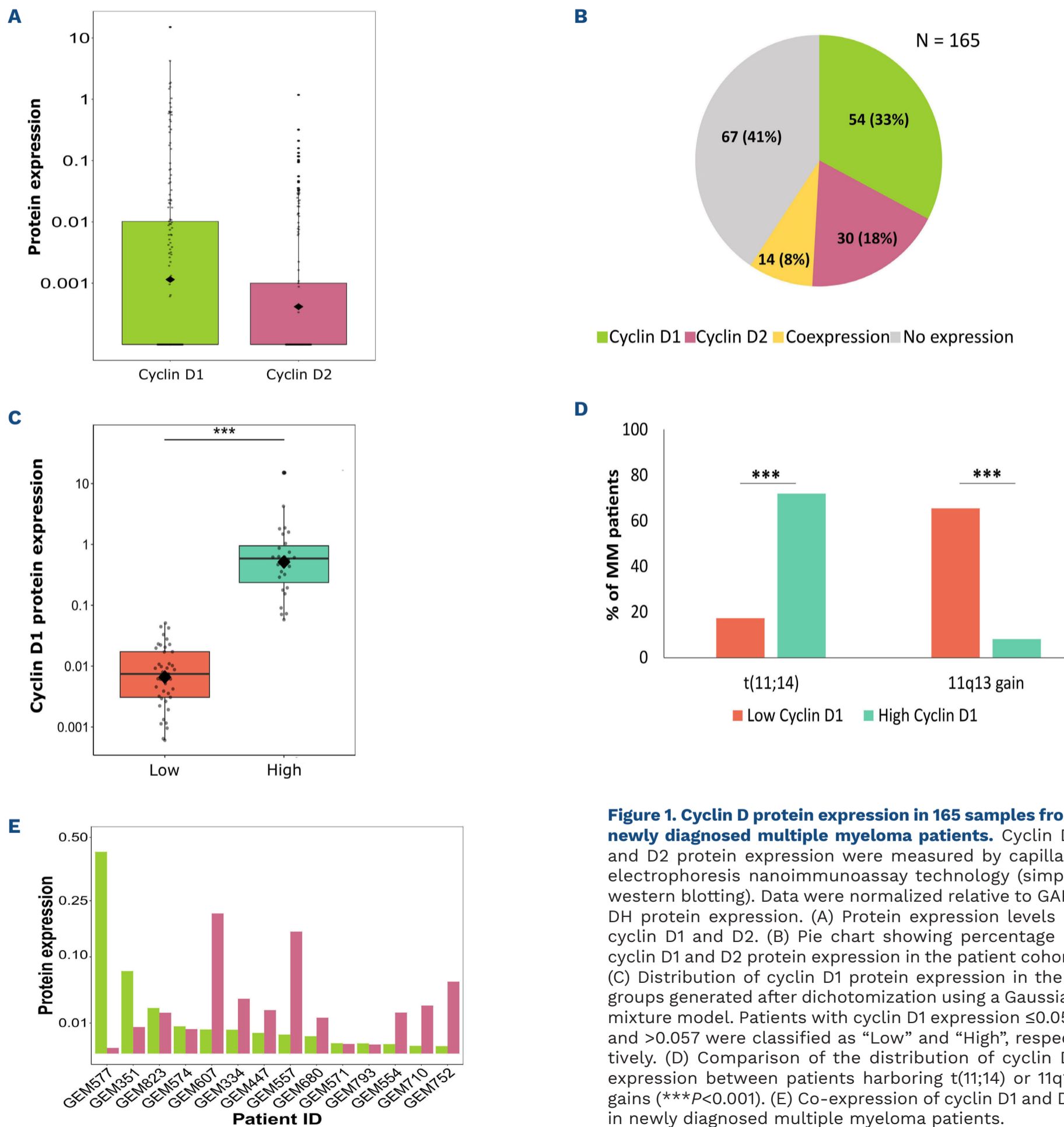
Quantifying cyclin D1 and D2 proteins showed a high proportion of MM patients without expression of any of the cyclins D. In order to gain more insight into this unexpected finding, we evaluated the expression of *CCND1* and *CCND2* at the mRNA level using qRT-PCR in 110 of the 165 samples

for which RNA was available.

Expression of *CCND1* and *CCND2* mRNA was quantified in 16 normal plasma cells (NPC) to establish the baseline expression level for both mRNA in the cohort. *CCND1* and *CCND2* mRNA were considered to be overexpressed when their expression in MM samples was above the upper 95th percentile expression level in NPC ($\Delta\text{Ct}=-5.99$ for *CCND1* and $\Delta\text{Ct}=-3.51$ for *CCND2*) (Figure 2A, B). According to these

criteria, exclusive overexpression of *CCND1* or *CCND2* was detected in 53% (58/110) and 21% (23/110) of patients, respectively. Overall, 6% (7/110) of the samples simultaneously expressed *CCND1* and *CCND2* at the mRNA level.

The Spearman's rank-order correlation between mRNA and protein expression levels was stronger for cyclin D1 than for cyclin D2 ($\rho=0.7$ vs. $\rho=0.53$; $P < 0.001$) (Online Supplementary Figure S2A, B).

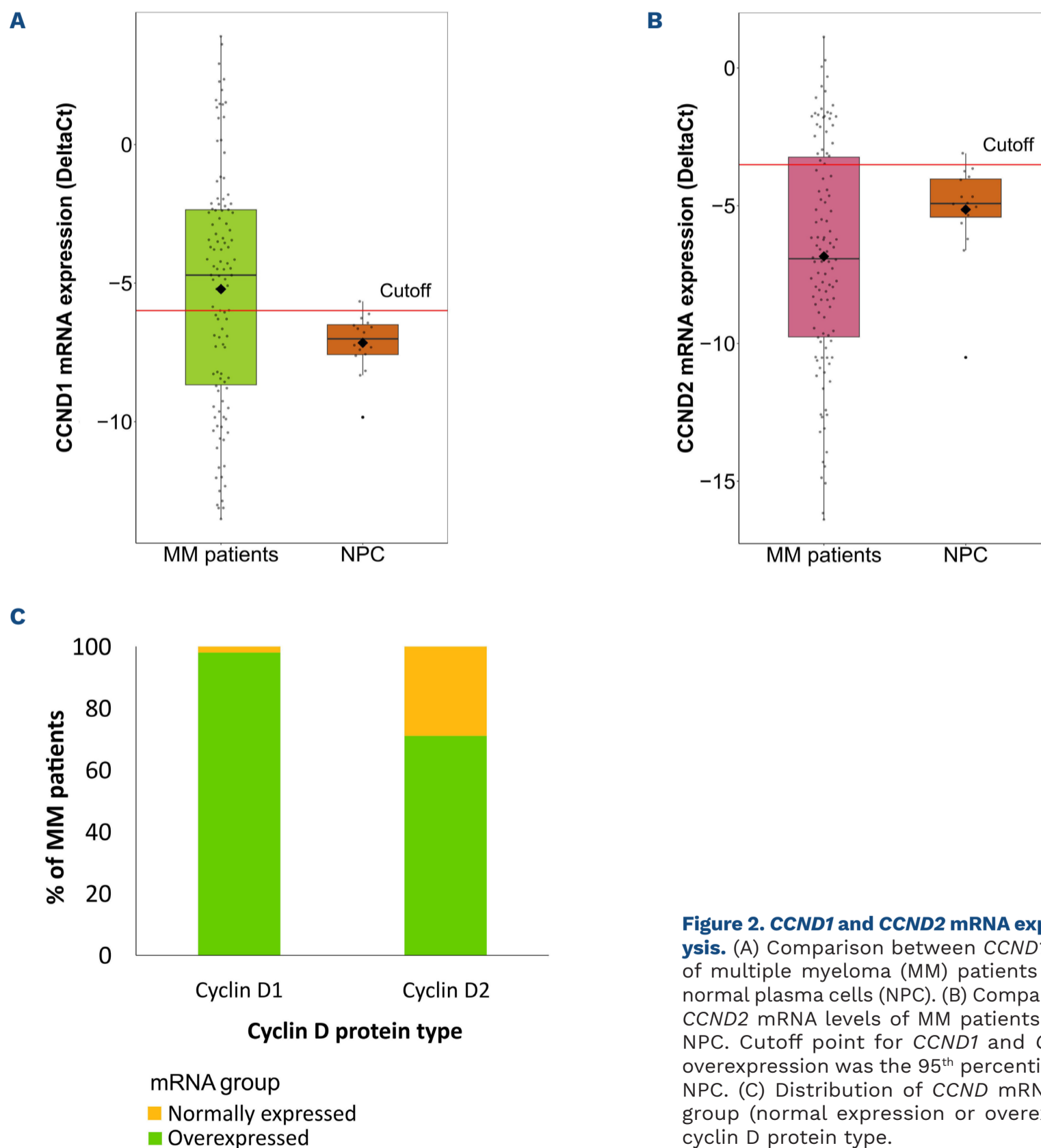


Almost all the samples that exclusively expressed cyclin D1 protein overexpressed *CCND1* mRNA (40/41 samples for which protein and mRNA material was available) (Figure 2C). The highest levels of *CCND1* mRNA were observed in MM patients with t(11;14).

However, when we compared the expression of cyclin D2 at the protein and mRNA levels in the samples for which both molecules were available, we found that 71% (15/21) of the patients exclusively expressing cyclin D2 protein also overexpressed *CCND2* mRNA (Figure 2C). Finally, the 41% of patients who did not express either cyclin D1 or cyclin D2 protein expressed mRNA at levels lower than those observed in NPC, whereas 59% of those patients expressed the mRNA of at least one cyclin D.

Prognostic effect of cyclin D protein expression

The survival analysis considered only the patients who exclusively expressed cyclin D1 or D2, and compared them with patients who did not express the corresponding cyclin D. Expression of cyclin D1 protein was significantly associated with longer OS (hazard ratio [HR] =0.44; 95% confidence interval [CI]: 0.22-0.91; $P=0.022$) (Figure 3A). Conversely, expression of cyclin D2 was significantly associated with shorter OS (HR=2.14; 95% CI: 1.13-4.05; $P=0.017$) (Figure 3B). No statistically significant differences were found in the TTP among the patients classified by their cyclin D1 or D2 expression status (Figure 3C, D). A positive effect of *CCND1* mRNA overexpression on OS was also observed (*Online Supplementary Figure S3*). Given the significant association between cyclin D2 protein



expression and 1q gains, we investigated how this relationship was related to survival. We found that the prognosis of patients with 1q gains was not affected by cyclin D2 protein levels, while cases expressing cyclin D2 exhibited short survival only if they also had 1q gains (Figure 4).

A subsequent survival analysis considering the groups of high and low expression of both cyclins D revealed no significant differences in OS between the two groups (Figure 5A). However, TTP was significantly shorter among patients with high levels of cyclin D1 (HR=2.43; 95% CI: 1.06-5.55; $P=0.03$), indicating a less favorable prognosis for patients with t(11;14) than for those with 11q gains (Figure 5B). Partitioning the patients into the high and low level cyclin D2 groups revealed no differential association with survival.

Discussion

Upregulation of D cyclins has been considered an early

initiating event in MM pathogenesis since one of the cyclin D genes is known to be overexpressed in almost all MGUS and MM patients.¹⁻³ These results were based on mRNA quantification using microarrays.^{1,3,18} Only limited attempts have been made to validate this overall finding at the protein level; the few studies carried out have only analyzed cyclin D1 protein by IHC in short series of patients.³²⁻³⁶ In this study, we quantified cyclin D1 and D2 proteins using CNIA in 165 newly diagnosed MM patients. Cyclin D3 was not included because of the very low frequency of MM cases overexpressing this cyclin D in previous analyses. We observed expression of the two cyclin D proteins, singly or together, in 59% of the patients. These results are in agreement with those of a previous analysis of cyclin D1 and D2 using IHC in almost 100 bone marrow biopsies, in which cyclin D1 protein was detected in 32%, cyclin D2 was found in 18% and both cyclins D were identified in 14% of MM patients.³⁶ Therefore, we did not detect any cyclin D expression in almost half of the MM samples, even using

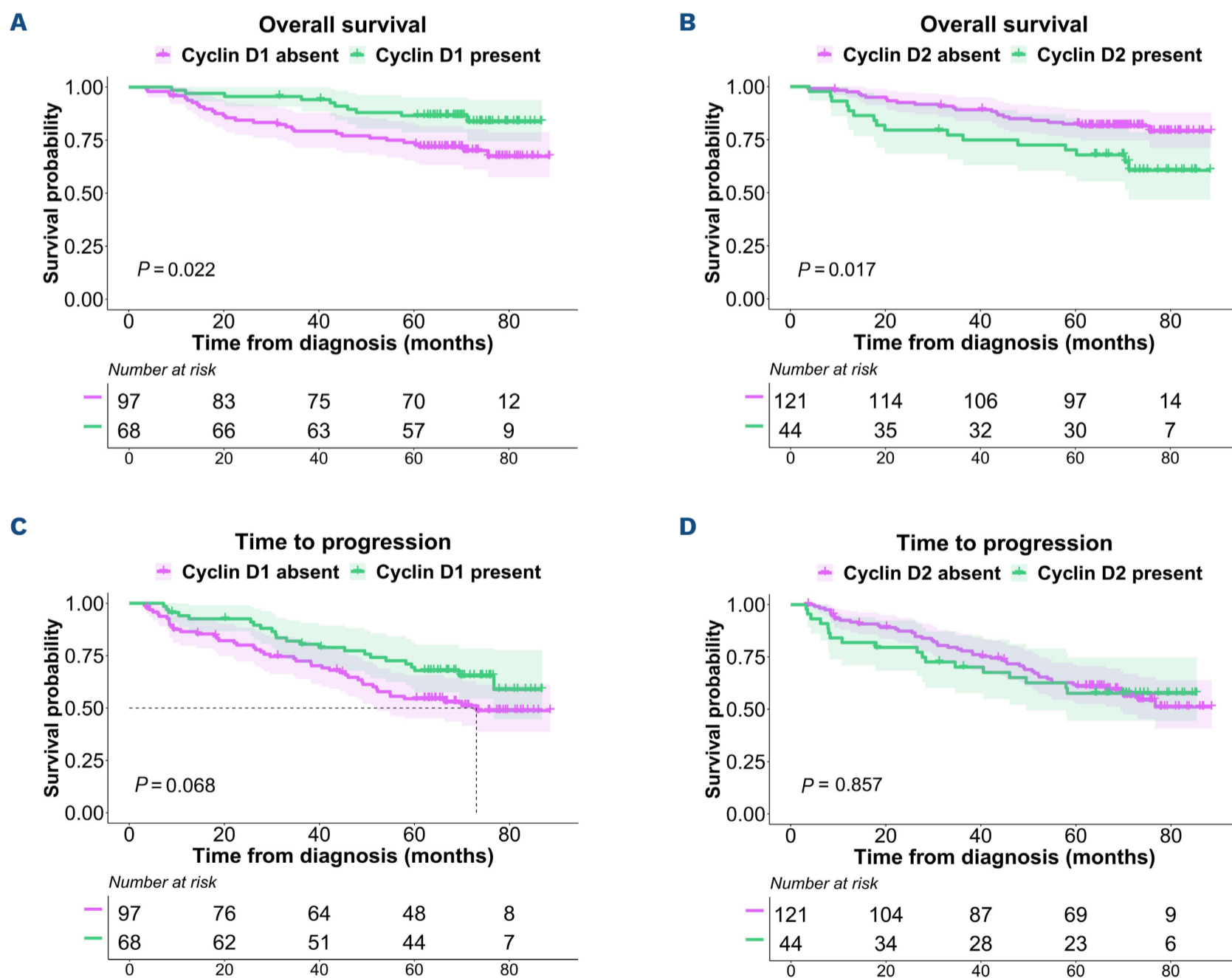


Figure 3. Impact of cyclin D1 and cyclin D2 protein expression on survival of multiple myeloma patients. (A, B) Kaplan-Meier curves of overall survival by cyclin D1 and cyclin D2 protein expression group, respectively. (C, D) Kaplan-Meier curves of time to progression by cyclin D1 and cyclin D2 protein expression group, respectively. Log-rank (Mantel-Cox) test P values are shown.

the CNIA method, which can accurately quantify proteins and is more sensitive than IHC. This finding prompted us to investigate *CCND1* and *CCND2* levels by RT-PCR, using the expression levels of both *CCND* in NPC as a cutoff to establish gene overexpression. It has been pointed out that *CCND1* is not expressed in NPC,^{1,18,37,38} and *CCND2*, is present at very low or null levels in NPC.^{1,18,39} We detected *CCND1* and *CCND2* overexpression in 53% and 21% of the patients, respectively, and simultaneous overexpression of *CCND1* and *CCND2* in a small group of patients. *CCND* genes were not expressed at levels above that of NPC in 20% of MM patients. This latter finding contrasts with the previously published results obtained using microarrays and RT-PCR, in which the proportion of MM patients not overexpressing cyclins D did not exceed 8%. Expression microarrays have

shown that *CCND1* and *CCND2* genes are both overexpressed in about 40-45% of MM patients, and that the other patients (approximately 11%) simultaneously express *CCND1* and *CCND2* or *CCND3*. These results were corroborated in other series of MM patients assessed using microarrays.^{1,18} Moreover, there was a very good concordance between cyclin D expression assessed by microarrays and RT-PCR.¹⁸ The fact that RT-PCR provides a relative quantification of mRNA may largely explain the differences between the percentage of MM patients who did not express D-cyclin mRNA in our study and in that of Agnelli's group.¹⁸ Protein expression of cyclins D in the present study also showed that cyclin D1 and cyclin D2 were overexpressed in an exclusive manner, and only a small proportion of patients coexpressed both cyclins D, as revealed by mRNA

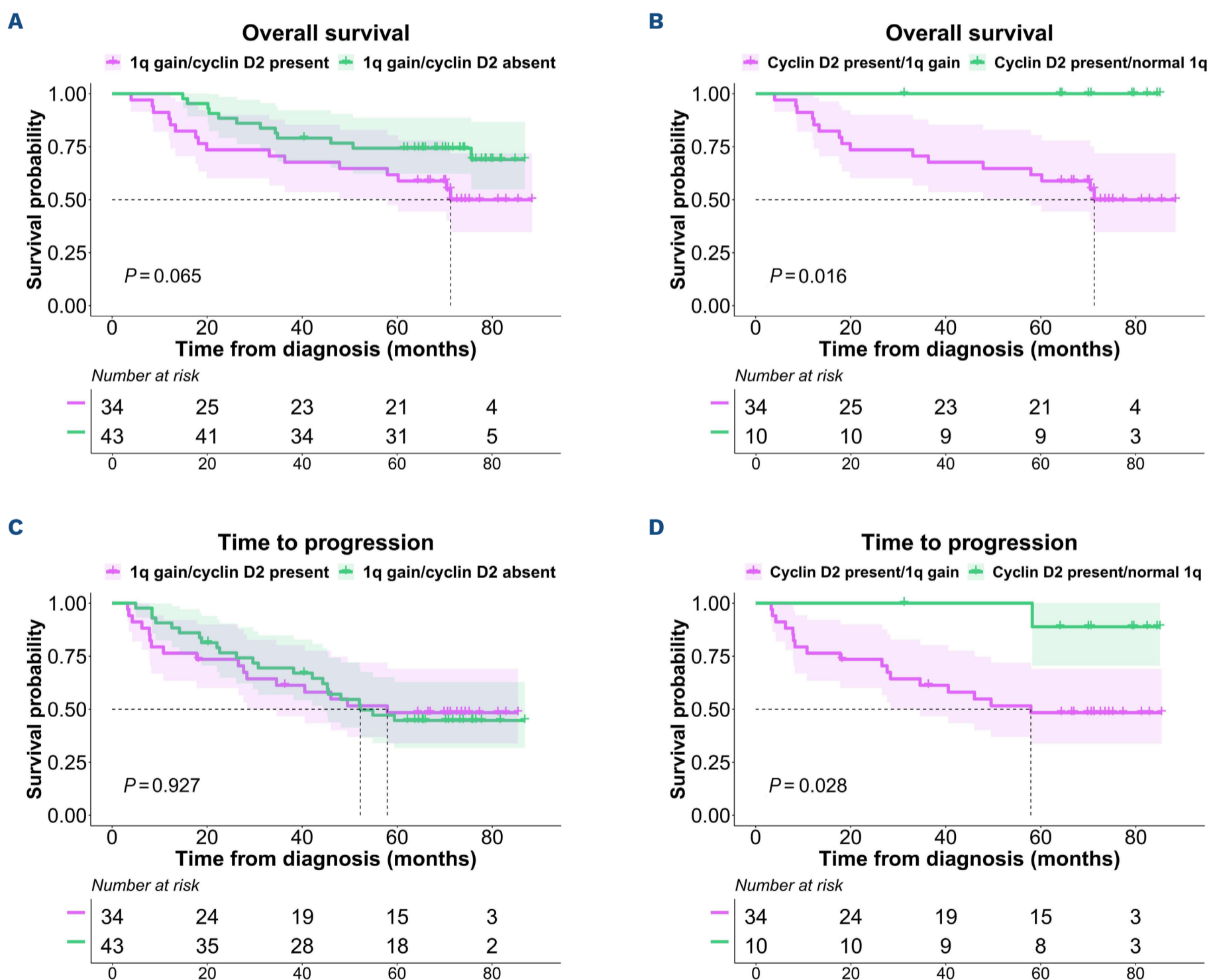


Figure 4. Survival analysis of the combination of cyclin D2 expression with 1q gain abnormality in multiple myeloma patients. (A, B) Kaplan-Meier curves of overall survival and time to progression (TTP), respectively, in patients with 1q gains according to the presence or absence of cyclin D2 protein. (C, D) Kaplan-Meier curves of overall survival and time to progression, respectively, in patients expressing cyclin D2 protein according to the presence or absence of 1q gains. Log-rank (Mantel-Cox) test P values are shown.

quantification^{1,3,18} and protein assays.³⁶

Our findings confirmed the strong association between the overexpression of cyclin D1 and the presence of t(11;14), as all the cases with this translocation overexpressed cyclin D1 protein, mostly at high levels. The patients overexpressing cyclin D1 at lower levels corresponded mainly to cases with 11q13 gains, although 37% of cases with this abnormality did not express cyclin D1 protein. As with the proteins, patients with high *CCND1* mRNA values had the t(11;14) translocation, and patients with 11q13 gain had intermediate levels of mRNA expression. These results are consistent with previous reports in which high levels and moderate levels of cyclin D1 mRNA were associated with t(11;14) and polysomy 11, respectively.^{1,3,5,26,40,41}

Overexpression of cyclin D2 may arise from different mechanisms that are not linked to translocations or amplification of *CCND2* gene.⁷⁻⁹ We found a significant association between cyclin D2 overexpression and t(14;16), 1q gain and 1p deletion, as described in particular in the case of t(14;16).^{1,3,7} However, 53% of patients with the t(4;14) translocation did not express cyclin D2. Even though the correlation between the protein and mRNA for the unique expression of cyclin D2 was weaker than that for cyclin D1, most of the samples expressing cyclin D2 protein also overexpressed *CCND2* mRNA. Six cases expressed cyclin D2 protein but with mRNA *CCND2* levels less than those found in NPC. This could be the result of the protein being generated by insignificant levels of *CCND2* mRNA.

Of the samples without cyclin D protein expression, the levels of mRNA expression of both *CCND1* and *CCND2* were less than the NPC cutoff in almost half of the patients, which explains the absence of protein. However, in the other patients one of the cyclins D was overexpressed at the mRNA level. This discrepancy could be related to post-transcriptional and post-translational modifications,

among other possible explanations.⁴²⁻⁴⁵ On the other hand, the greater sensitivity of qRT-PCR compared to the CNIA technique could explain why some cases in which protein expression was not observed, the corresponding mRNA was detected. However, mRNA levels cannot be considered as the final output of gene expression, while proteins are closer to phenotypes and to gene function.⁴⁶

Survival analysis showed that OS was significantly shorter for patients expressing cyclin D2 protein, while high levels of cyclin D1 protein were associated with prolonged OS. These results are consistent with those previously published, which demonstrate a significantly better prognosis for the patients who expressed high levels of cyclin D1 protein detected by immunohistochemistry than for those with low or null levels of cyclin D1 expression.^{27,47} Overexpression of *CCND1* mRNA has also been associated with better prognosis.³ The different effect on OS depending on the levels of cyclin D1 and cyclin D2 was not observed for TTP in the present series, indicating the effectiveness of VRD induction and ASCT consolidation in all patients independently of the level of expression of cyclin D proteins. However, the strong association between the expression of cyclin D1 and t(11;14) and polysomy 11, and between the expression of cyclin D2 and the presence of high-risk cytogenetic abnormalities suggests that the differences in survival for each cyclin D are related to cytogenetic abnormalities rather than to cyclin D expression.^{3,39} In fact, patients with cyclin D2 protein expression but without 1q gains had a favorable prognosis.

When the survival analysis partitioned the cyclin D1 expression into high and low levels, we found that patients with high levels of cyclin D1 protein had significantly shorter TTP than did those with low levels, although this difference was not maintained during the subsequent course of the disease, since OS was similar for both groups. The strong

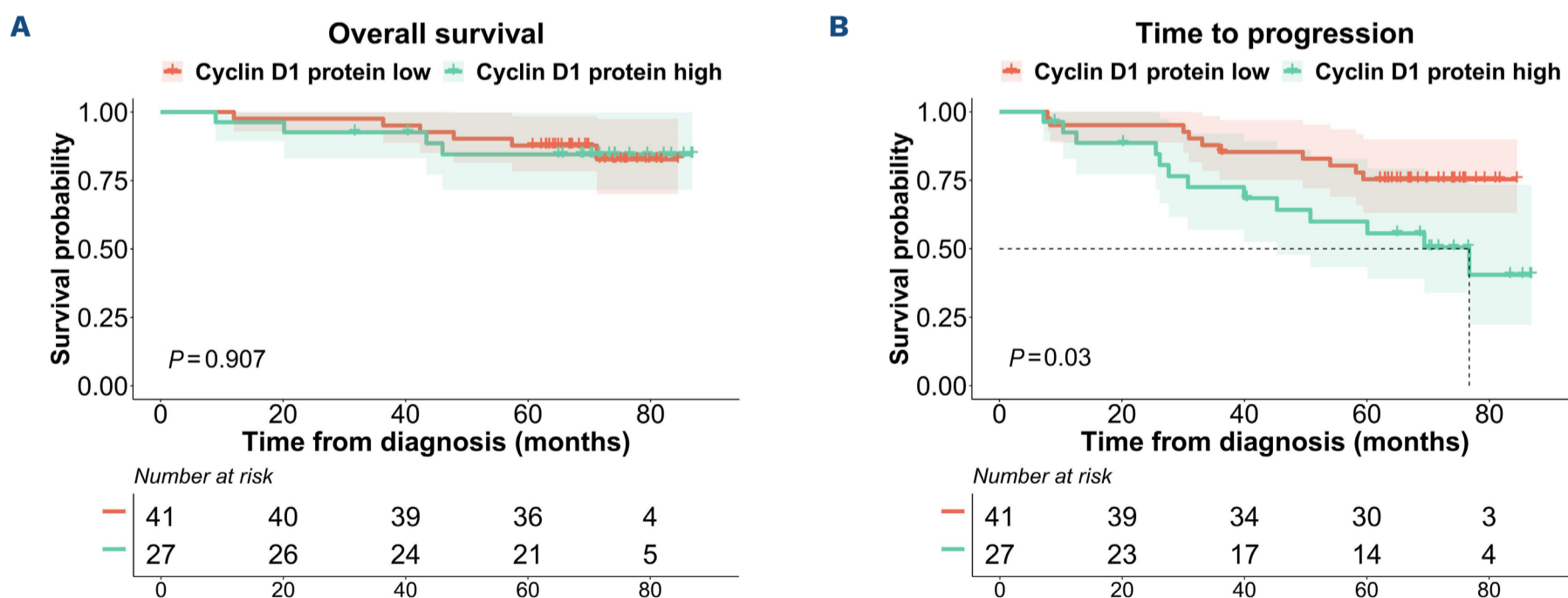


Figure 5. Survival analysis by dichotomized cyclin D1 protein expression groups (high and low). (A and B) Kaplan-Meier curves for overall survival and time to progression, respectively. Log-rank (Mantel-Cox) test P values are shown.

association between lower cyclin D levels and 11q13 gains indicates a more favorable outcome for MM patients with 11q13 than for those with t(11;14). This is consistent with the findings of earlier studies.^{48,49}

In summary, no cyclin D1 or D2 protein expression was detected in about half of the MM patients, in whom 41% of the cases could be explained by very low mRNA levels (values less than the NPC cutoff). The discrepancy between cyclin D protein abundance and mRNA levels in the other cases may be related to post-transcriptional and post-translational mechanisms. In any case, our data demonstrate that D cyclin proteins are not universally expressed in MM. While we found that cyclin D1 was overexpressed in almost all cases with t(11;14) and 11q13 gain, cyclin D2 was not detected in the majority of the MM patients not expressing cyclin D1. Although GEP had demonstrated dysregulation of one of the D cyclins at the mRNA level in almost all MM, this increased expression does not culminate in the production of more protein, especially in the case of cyclin D2. This suggests that cyclins D could not play an oncogenic role in a proportion of patients with MM. On the other hand, it remains to be determined whether the high levels of cyclin D present in approximately 60% of patients with MM are always functionally relevant. In terms of the prognostic impact of cyclins D, our results support that the relationship of their overexpression with the prognosis of patients with MM is driven more by genetic alterations associated with cyclin D1 and D2 upregulation than by their dysregulation *per se*.

Disclosures

LR has received consulting fees from Amgen, BMS/Celgene, Sanofi, Janssen, Takeda, GSK and Karyofarm. JML has received honoraria and consulting fees from BMS/Celgene, Incyte, Janssen, Novartis, Sanofi and Roche. JSM has consulting and advisory roles with Amgen, Bristol-Myers Squibb, Celgene, Janssen, MSD, Novartis, Takeda, and Roche. MVM served on speakers bureaus and advisory boards for AbbVie, Adaptive, Amgen, Celgene, GlaxoSmithKline, Janssen, Mundipharma, Oncopeptides, PharmaMar, Roche, Seattle Genetics, and Takeda. NCG receives honoraria from Janssen and Amgen. All the other authors have no conflicts of interest to disclose.

References

- Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*. 2005;106(1):296-303.
- Bergsagel PL, Kuehl WM. Molecular pathogenesis and a consequent classification of multiple myeloma. *J Clin Oncol*. 2005;23(26):6333-6338.
- Zhan F, Huang Y, Colla S, et al. The molecular classification of multiple myeloma. *Blood*. 2006;108(6):2020-2028.
- Skerget S, Penaherrera D, Chari A, et al. Genomic basis of multiple myeloma subtypes from the MMRF CoMMpass Study. medRxiv. 2021 Aug 5. doi: 10.1101/2021.08.02.21261211 [preprint, not peer-reviewed].
- Soverini S, Cavo M, Cellini C, et al. Cyclin D1 overexpression is a favorable prognostic variable for newly diagnosed multiple myeloma patients treated with high-dose chemotherapy and single or double autologous transplantation. *Blood*. 2003;102(5):1588-1594.
- Lesage D, Troussard X, Sola B. The enigmatic role of cyclin D1 in

Contributions

IJCB developed and performed qRT-PCR, analyzed data, prepared the figures and wrote the manuscript. IMK conceived the idea, developed and performed CNIA experiments and analyzed protein data. EAR performed CNIA experiments and analyzed protein data. CDR obtained the patients' clinical data. II assisted with CNIA experiments. ASS and DQ assisted with qRT-PCR experiments. AMLG and MC assisted with laboratory experiments. MJC, LR, JML, JSM and MVM provided patient samples and clinical data, and were responsible for obtaining informed consent from patients. LAC analyzed the clinical data, supervised the statistical analysis, helped prepare the figures and supervised the whole study. NCG conceived the idea and designed the study, participated in writing the manuscript, supervised the whole study, and provided funding. All authors critically reviewed and approved the manuscript.

Acknowledgments

The authors thank Vanesa Gutiérrez for her technical assistance with MM cell purification and FISH analysis, and Phil Mason for his help in reviewing the English language of the manuscript.

Funding

This study was funded by the Instituto de Salud Carlos III and co-financed by FEDER (PI16/01074 and PI19/00674); by the Asociación Española Contra el Cáncer (AECC) (Proyectos Estratégicos: PROYE20047GUTI); and by the Gerencia Regional de Salud, Junta de Castilla y León grants (GRS1654/A/17, GRS1849/A/18, and GRS2058/A/19). IJCB was supported by a fellowship (contract PFIS-2020: FI20/00226) from the Instituto de Salud Carlos III (contract PFIS-2020: FI20/00226). CDR was supported by a fellowship from the AECC (CL-JUN18010DERA); EARR was supported by the Consejería de Educación de Castilla y León and FEDER funds. The WES platform was acquired thanks to the INNOCAMPUS program (CE110-1-0010).

Data-sharing statement

The original data and protocols used for this article can be acquired by writing to the first author (icarbe96@usal.es) or the corresponding author.

- multiple myeloma. *Int J Cancer*. 2005;115(2):171-176.
7. Hurt EM, Wiestner A, Rosenwald A, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell*. 2004;5(2):191-199.
 8. Yang L, Wang H, Kornblau SM, et al. Evidence of a role for the novel zinc-finger transcription factor ZKSCAN3 in modulating Cyclin D2 expression in multiple myeloma. *Oncogene*. 2011;30(11):1329-1340.
 9. Misiewicz-Krzeminska I, Sarasquete ME, Vicente-Dueñas C, et al. Post-transcriptional modifications contribute to the upregulation of cyclin D2 in multiple myeloma. *Clin Cancer Res*. 2016;22(1):207-217
 10. Montalto FI, De Amicis F. Cyclin D1 in cancer: a molecular connection for cell cycle control, adhesion and invasion in tumor and stroma. *Cells*. 2020;9(12):2648.
 11. Tiedemann RE, Mao X, Shi C-X, et al. Identification of kinetin riboside as a repressor of CCND1 and CCND2 with preclinical antimyeloma activity. *J Clin Invest*. 2008;118(5):1750-1764.
 12. Barwick BG, Gupta VA, Vertino PM, Boise LH. Cell of origin and genetic alterations in the pathogenesis of multiple myeloma. *Front Immunol*. 2019;10:1121.
 13. Hydring P, Malumbres M, Sicinski P. Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases. *Nat Rev Mol Cell Biol*. 2016;17(5):280-292.
 14. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer*. 2011;11(8):558-572.
 15. Comstock CES, Augello MA, Benito RP, et al. Cyclin D1 splice variants: polymorphism, risk, and isoform-specific regulation in prostate cancer. *Clin Cancer Res*. 2009;15(17):5338-5349.
 16. Tchakarska G, Sola B. The double dealing of cyclin D1. *Cell Cycle*. 2020;19(2):163-178.
 17. Dai J, Wei R-J, Li R, Feng J-B, Yu Y-L, Liu P-S. A study of CCND1 with epithelial ovarian cancer cell proliferation and apoptosis. *Eur Rev Med Pharmacol Sci*. 2016;20(20):4230-4235.
 18. Agnelli L, Bicciato S, Mattioli M, et al. Molecular classification of multiple myeloma: a distinct transcriptional profile characterizes patients expressing CCND1 and negative for 14q32 translocations. *J Clin Oncol*. 2005;23(29):7296-7306.
 19. Ding Z-Y, Li R, Zhang Q-J, et al. Prognostic role of cyclin D2/D3 in multiple human malignant neoplasms: A systematic review and meta-analysis. *Cancer Med*. 2019;8(6):2717-2729.
 20. Cardona-Benavides IJ, de Ramón C, Gutiérrez NC. Genetic abnormalities in multiple myeloma: prognostic and therapeutic implications. *Cells*. 2021;10(2):336.
 21. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer*. 2007;7(8):585-598.
 22. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2014;15(12):e538-548.
 23. Fonseca R, Bergsagel PL, Drach J, et al. International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. *Leukemia*. 2009;23(12):2210-2221.
 24. Avet-Loiseau H, Durie BGM, Cavo M, et al. Combining fluorescent in situ hybridization data with ISS staging improves risk assessment in myeloma: an International Myeloma Working Group collaborative project. *Leukemia*. 2013;27(3):711-717.
 25. Quinn J, Glassford J, Percy L, et al. APRIL promotes cell-cycle progression in primary multiple myeloma cells: influence of D-type cyclin group and translocation status. *Blood*. 2011;117(3):890-901.
 26. Specht K, Kremer M, Müller U, et al. Identification of cyclin D1 mRNA overexpression in B-cell neoplasias by real-time reverse transcription-PCR of microdissected paraffin sections. *Clin Cancer Res*. 2002;8(9):2902-2911.
 27. Dawson MA, Opat SS, Taouk Y, et al. Clinical and immunohistochemical features associated with a response to bortezomib in patients with multiple myeloma. *Clin Cancer Res*. 2009;15(2):714-722.
 28. Misiewicz-Krzeminska I, Corchete LA, Rojas EA, et al. A novel nano-immunoassay method for quantification of proteins from CD138-purified myeloma cells: biological and clinical utility. *Haematologica*. 2018;103(5):880-889.
 29. Misiewicz-Krzeminska I, Isidro I, Gutiérrez NC. Capillary nano-immunoassay for quantification of proteins from CD138-purified myeloma cells. *Bio-Protoc*. 2019;9(12):e3267.
 30. Rosiñol L, Oriol A, Rios R, et al. Bortezomib, lenalidomide, and dexamethasone as induction therapy prior to autologous transplant in multiple myeloma. *Blood*. 2019;134(16):1337-1345.
 31. Misiewicz-Krzeminska I, de Ramón C, Corchete LA, et al. Quantitative expression of Ikaros, IRF4, and PSMD10 proteins predicts survival in VRD-treated patients with multiple myeloma. *Blood Adv*. 2020;4(23):6023-6033.
 32. Kremer M, Ott G, Nathrath M, et al. Primary extramedullary plasmacytoma and multiple myeloma: phenotypic differences revealed by immunohistochemical analysis. *J Pathol*. 2005;205(1):92-101.
 33. Athanasiou E, Kaloutsi V, Kotoula V, et al. Cyclin D1 overexpression in multiple myeloma. A morphologic, immunohistochemical, and in situ hybridization study of 71 paraffin-embedded bone marrow biopsy specimens. *Am J Clin Pathol*. 2001;116(4):535-542.
 34. Padhi S, Varghese RG, Ramdas A. Cyclin D1 expression in multiple myeloma by immunohistochemistry: Case series of 14 patients and literature review. *Indian J Med Paediatr Oncol*. 2013;34(4):283-291.
 35. Markovic O, Marisavljevic D, Cemerikic V, Suvajdzic N, Milic N, Colovic M. Immunohistochemical analysis of cyclin D1 and p53 in multiple myeloma: relationship to proliferative activity and prognostic significance. *Med Oncol*. 2004;21(1):73-80.
 36. Mansoor A, Akhter A, Pournazari P, et al. Protein expression for novel prognostic markers (Cyclins D1, D2, D3, B1, B2, ITGβ7, FGFR3, PAX5) correlate with previously reported gene expression profile patterns in plasma cell myeloma. *Appl Immunohistochem Mol Morphol*. 2015;23(5):327-333.
 37. Zhan F, Hardin J, Kordsmeier B, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. *Blood*. 2002;99(5):1745-1757.
 38. De Vos J, Thykjaer T, Tarte K, et al. Comparison of gene expression profiling between malignant and normal plasma cells with oligonucleotide arrays. *Oncogene*. 2002;21(44):6848-6857.
 39. Hanamura I, Huang Y, Zhan F, Barlogie B, Shaughnessy J. Prognostic value of cyclin D2 mRNA expression in newly diagnosed multiple myeloma treated with high-dose chemotherapy and tandem autologous stem cell transplantations. *Leukemia*. 2006;20(7):1288-1290.
 40. Specht K, Haralambieva E, Bink K, et al. Different mechanisms of cyclin D1 overexpression in multiple myeloma revealed by fluorescence in situ hybridization and quantitative analysis of mRNA levels. *Blood*. 2004;104(4):1120-1126.
 41. Pruneri G, Fabris S, Baldini L, et al. Immunohistochemical

- analysis of cyclin D1 shows deregulated expression in multiple myeloma with the t(11;14). *Am J Pathol.* 2000;156(5):1505-1513.
42. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* 2012;13(4):227-232.
43. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell.* 2016;165(3):535-550.
44. Qie S, Diehl JA. Cyclin D1, cancer progression, and opportunities in cancer treatment. *J Mol Med.* 2016;94(12):1313-1326.
45. Simoneschi D, Rona G, Zhou N, et al. CRL4AMBRA1 is a master regulator of D-type cyclins. *Nature.* 2021;592(7856):789-793.
46. Buccitelli C, Selbach M. mRNAs, proteins and the emerging principles of gene expression control. *Nat Rev Genet.* 2020;21(10):630-644.
47. Cook JR, Hsi ED, Worley S, Tubbs RR, Hussein M. Immunohistochemical analysis identifies two cyclin D1+ subsets of plasma cell myeloma, each associated with favorable survival. *Am J Clin Pathol.* 2006;125(4):615-624.
48. Gran C, Uttervall K, Borg Bruchfeld J, et al. Translocation (11;14) in newly diagnosed multiple myeloma, time to reclassify this standard risk chromosomal aberration? *Eur J Haematol.* 2019;103(6):588-596.
49. Joseph NS, Kaufman JL, Dhodapkar MV, et al. Long-term follow-up results of lenalidomide, bortezomib, and dexamethasone induction therapy and risk-adapted maintenance approach in newly diagnosed multiple myeloma. *J Clin Oncol.* 2020;38(17):1928-1937.