Impact of MiRSNPs on Survival and Progression in Patients with Multiple Myeloma Undergoing Autologous Stem Cell Transplantation

Carlos Fernández de Larrea¹, Alfons Navarro², Rut Tejero², Natalia Tovar¹, Tania Díaz², María Teresa Cibeira¹, Laura Rosiño³, Gerardo Ferrer², Montserrat Rovira¹, Maria Rozman³, Mariano Monzo², and Joan Bladé¹

Abstract

**Purpose:** A distinctive new group of polymorphisms is constituted by single-nucleotide polymorphism (SNP) in miRNA processing machinery in miRNA precursor molecules and in miRNA-binding sites, known as miRSNPs. The aim of this study was to ascertain the prognostic impact of six miRSNPs in patients with multiple myeloma and analyze the functional consequences.

**Experimental Design:** One hundred and thirty-seven patients with chemosensitive multiple myeloma (73M/64F) intensified with autologous stem cell transplantation (ASCT) were studied. The median follow-up was 4 years. The genes and SNPs evaluated in genomic DNA by allelic discrimination were KRT81 (rs3660), AFF1 (rs17703261), FAM179b (rs1053667), and MIR196A2 (rs11614913) for miRNA target genes and TRBP (rs784567) and XPO5 (rs11077) for miRNA biogenesis pathway.

**Results:** Overall survival (OS) was significantly longer in patients with KRT81 rs3660 C/C variant ($P = 0.037$). Functional analysis showed that the presence of C variant in KRT81 3’ untranslated region (UTR) is related with a reduction of the protein levels. Moreover, the reduction of KRT81 protein levels by siRNA in multiple myeloma cell lines is related to a decreased proliferation. On the other hand, OS was significantly longer in patients with C/C or A/C variant in XPO5 rs11077 ($P = 0.012$). There was also a significantly longer progression-free survival (PFS) for this SNP ($P = 0.013$). This SNP retained its prognostic impact on PFS and OS in a multivariate regression analysis ($P = 0.028$ and $P = 0.014$, respectively).

**Conclusion:** This is the first report that relates miRSNPs with prognosis in multiple myeloma either in a keratin gene (KRT81), target of diverse miRNA multiple myeloma clusters, or in the miRNA biogenesis pathway–related protein exportin-5. *Clin Cancer Res;* 1–8. ©2012 AACR.
myeloma (9, 10), no studies have been reported to date with miRSNPs in multiple myeloma. This novel class of SNPs opens a new area of research in cancer biology and clinical oncology.

The aim of this study was to ascertain the prognostic impact of 6 miRSNPs located either in multiple myeloma–related miRNAs target genes or in miRNA biogenesis pathway proteins in multiple myeloma undergoing ASCT. We tested this hypothesis in a homogeneous population of 137 patients with chemosensitive multiple myeloma who had undergone ASCT as part of the first-line therapy in a single institution.

Materials and Methods

Patient recruitment and data collection

From March 31, 1994, to November 16, 2010, 192 patients with multiple myeloma underwent ASCT in our institution. One hundred and fifty-eight (82.3%) of them had chemosensitive disease at the time of ASCT. Of these, 137 patients (74M/63F; median age, 55 years; range 26–67 years) had a genomic DNA sample available and they were the subjects of the present investigation. The induction regimen more frequently used was the standard combination chemotherapy (99 patients, 72.3%), mainly alternat-

Translational Relevance

This is the first study reporting that polymorphisms related to miRSNPs have prognostic value in multiple myeloma. Two miRSNPs, one in a keratin gene (KRT81), and other in the miRNA biogenesis pathway–related protein exportin-5 (XPO5), can modify the final levels of these proteins and are involved in the prognosis after melphalan-based autologous stem cell transplantation of patients with multiple myeloma. This information could be helpful to incorporate additional measures in the first-line therapy before autologous stem cell transplantation, according to the genetic background.

Table 1. Patient characteristics (N = 137)

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DNA extraction and genotyping

Genomic DNA was isolated from bone marrow aspirate slides or peripheral blood using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s instructions. All DNA samples were stored at −80°C, suspended in Tris-EDTA (TE) buffer. After a selection based on the reported association of the selected miRSNPs with cancer risk (8, 12, 13), a group of genes and SNPs were selected: KRT81 (rs3660), AFFI (rs17703261), FAM179b (rs1053667), MIR196A2 (rs11614913), TRBP (rs784567), and XPO5 (rs11077). Inclusion criteria for each SNP included a minor genotype frequency for the European population of 0.05 or more and either a known association with a differential susceptibility to cancer development or a differential impact in patients with solid tumors. Previous reports of our group of prognosis association of some of these miRSNPs in lung cancer (14) were also taken into account. SNP analysis was conducted by allelic discrimination with TaqMan probes (commercial assays) in an ABI PRISM 7500 Sequence detection system (Applied Biosystems) as previously described (15). At least 10% of the samples were studied by PCR in the second time, obtaining the same genotypic results. All samples had a code number at the time of extraction, as guarantee of the blinded analysis.

mRNA and protein analysis

Total RNA was extracted from lymphocytes from peripheral blood of healthy donors using TRIzol (Invitrogen) according to the manufacturer’s protocol. Total cDNA was...
synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer’s protocol. Quantitative PCR (qPCR) was carried out using TaqMan Gene Expression Assays (Applied Biosystems) for XPO5 (Hs00382455_m1), KRT81 (Hs00605559_m1), and GUSB (Hs99999908_m1) used as endogenous control.

Total protein was isolated from lymphocytes from peripheral blood of healthy donors using Qiagen ProteinPrep Kit (Qiagen) according to the manufacturer's protocol. XPO5 and KRT81 protein levels were determined using exportin-5 (XPO5) and keratin 81 (KRT81) ELISA Kit (antibodies online GmbH) as per manufacturer’s protocol.

**Cell culture and transfection**

The multiple myeloma cell lines KMM-1 and RPMI-8226 were cultured in RPMI-1640 containing 20% and 10% fetal calf serum (FCS; Invitrogen), respectively. These cell lines were obtained from Hematopathology Unit (Dolors Colomer, PhD, Hospital Clinic of Barcelona, University of Barcelona, Barcelona, Spain) just before the experiment. Both cell lines were transfected with 33 nmol/L of keratin 81 siRNA (sc-95778) and control si-RNA A (sc-37007A; Santa Cruz Biotechnology, Inc.) by using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol with minor modifications. Briefly, 1 hour before transfection, both cell lines were counted and 250,000 cells per well were diluted in 50 μL of Opti-MEM (Invitrogen) without serum and plated in coated 96-well plates. Later, the siRNA and Lipofectamine 2000 complexes diluted in 50 μL of Opti-MEM were added to the cells and subsequently incubated at 37°C in 5% CO2 for 4 hours without serum. Fifty microliters of RPMI-1640 with FCS was then added for further incubation. All the experiments were carried out in triplicate.

**Proliferation assays**

Proliferation in KMM-1 and RPMI-8226 cells transfected with keratin 81 siRNA or with control si-RNA A were directly measured at 24 hours after transfection using a CellTititer 96 AQueous One Solution Cell Proliferation Assay (MTS) as per the manufacturer’s protocol. Cell proliferation was assessed by measuring the absorbance at 490 nm with a Promega Dual Luciferase Reporter Assay System (Promega) in a Veritas microplate luminometer. The transfection efficiency was normalized with the firefly luciferase gene.

**Renilla/luciferase reporter assays**

Eight synthetic oligonucleotides, containing from 5’ to 3’: XhoI sticky end (5 bp), a fragment from the 3’ untranslated region (UTR) of KRT81 gene containing the wild-type (WT) or the SNP sequence (rs3660; 41 bp) or a fragment from the 3’ UTR of XPO5 gene containing the WT or the SNP sequence (rs11077; 47 bp), BglII restriction site (6 bp), and NotI sticky end (2 bp), were cloned in the psiCheck2 vector (Promega) in the 3’UTR of Renilla luciferase gene. The 2 oligonucleotides for KRT81-WT sense (5’-TCGAGAGTGCTCAGCTACTTC-3’), and antisense (5’-CCCTAGATCTGC-3’), the 2 for KRT81-SNP sense (5’-TGAGAGTGCTCAGCTACTTC-3’), and antisense (5’-CGCCAGATCGTGGAGGTAC-3’), and the 2 oligonucleotides for XPO5-WT sense (5’-TGAGAGTACCCTCAGAGAGAAGTGC-3’), and antisense (5’-GCCGAGATCGTGGAGGTAC-3’), and the 2 oligonucleotides for XPO5-SNP sense (5’-TGAGTACCCTCAGAGAGAAGTGC-3’) and antisense (5’-GCCGAGATCGTGGAGGTAC-3’). The 2 were first annealed with a Tris buffer (100 mmol/L Tris-HCl, pH 7.5, 1 mol/L NaCl, 10 mmol/EDTA) in a heating block at 95°C for 5 minutes, followed by a gradual reduction of temperature until room temperature. The psiCheck2 vector was linearized by digestion with NotI and XhoI (New England Biolabs) and purified from an agarose gel. The annealed oligonucleotides were ligated in the linearized psiCheck2 vector into the modified psiCheck2 vector containing the insert with the SNP (SNP-modified psiCheck2) or the modified psiCheck2 vector into the NotI and XhoI cloning sites located in the downstream of the Renilla luciferase reporter gene with T4 DNA ligase (New England Biolabs). The ligation reaction was transformed in TOP10 F’ Escherichia coli competent cells (Invitrogen). Positive clones were selected by restriction digestion with BglII and confirmed by sequencing.

The 2 cell lines were transfected with 0.5 μg of either the modified psiCheck2 vector containing the insert with the WT sequence (WT-modified psiCheck2) or the modified psiCheck2 vector containing the insert with the SNP (SNP-modified psiCheck2). Then, the Renilla luciferase and firefly luciferase activity was measured 48 hours after transfection with the Promega Dual Luciferase Reporter Assay System (Promega) in a Veritas microplate luminometer. The transfection efficiency was normalized with the firefly luciferase gene.

**Statistical methods**

Statistical analyses were conducted with PAWS statistics software 18.0 for Windows and GraphPad 5. PFS was defined as survival from ASCT until relapse or death from any cause. OS was calculated from the time of ASCT to last follow-up or death. Survival probabilities were estimated using the Kaplan–Meier method and analyzed by means of the log-rank test. Cox proportional hazards model was used to estimate the risk ratio of events (relative risk, RR) with the respective confidence interval (CI), and to multivariate analysis. The t test was used to compare differential expression between genotypic groups. The genotypes were combined for statistical reasons when different biologic plausible combinations were analyzed, and 2 of them showed an overlapped trend. Statistical significance was defined as P < 0.05.

**Results**

MiRSNPs in Myeloma

In the overall population, the median PFS from ASCT was 2.8 years (95% CI, 2–3.7 years), with a median OS of 9.7
years (95% CI, 5.2–14.2 years). Two miRSNPs had prognostic impact after ASCT. The first one, rs3660 in KRT81 gene, was associated with a significantly longer OS in patients with CC (21) versus CG or GG genotype (111; median not reached vs. 7 years; \( P = 0.037 \); RR, 3.26; 95% CI, 1.1–10.5; Fig. 1A). Only 3 late deaths were observed in the favorable group with 21 patients at risk. When only patients who underwent ASCT after first-line treatment were considered, this significance was retained (\( P = 0.046 \)).

The same variants in this miRSNP showed a longer PFS (median 4.3 vs. 2.4 years) but not reaching statistical significance (\( P = 0.24 \)). The second miRSNP was rs11077 in XPO5. Patients with CC/AC genotype (\( n = 86 \)) had a significantly longer OS than patients with AA genotype (31; median not reached vs. 7.2 years; \( P = 0.012 \); RR, 2.5; 95% CI, 1.2–5; Fig. 1B). Interestingly, and in the same line, patients with CC/AC genotype showed a higher PFS (median, 4.3 vs. 2 years; \( P = 0.013 \); RR 1.97; 95% CI, 1.12–3.4; Fig. 2).

The most unfavorable variant (AA) showed also a significantly shorter PFS when compared with the 2 other genotypes (AC, \( P = 0.032 \); CC, \( P = 0.023 \)). The complete response (CR) rate was not significantly different among the different genotype groups. When only patients who received ASCT after first-line treatment were considered, the effect over OS was even deeper (\( P = 0.005 \); RR 2.9; 95% CI, 1.3–6.4). Furthermore, the effect on OS was also significant in the subset of patients achieving CR after ASCT (\( P = 0.03 \)).

The miRSNP rs784567 in TRBP gene showed a trend toward a shorter OS (\( P = 0.103 \)) and PFS (\( P = 0.083 \)) in the same sense. All the patients were heterozygous to AFF1 (rs17703261), so no further analysis was conducted on this SNP. No associations with prognosis or toxicities were observed with the other SNPs studied.

The main baseline clinical and laboratory characteristics of the patients were not significantly different between these genotypic groups. The incorporation of new drugs (thalidomide and bortezomib) into the induction regimens before ASCT according to the genotypes with clinical difference was no different neither in KRT81 rs3660 (26.8% vs. 28.7%; \( P = 0.884 \)), nor XPO5 rs11077 (22.1% vs. 32.3%; \( P = 0.332 \)). There was no significant difference in pretransplant response between the 2 genotypes neither in KRT81 rs3660 (\( P = 0.409 \)) nor XPO5 rs11077 (\( P = 0.915 \)). In fact, CR rate in both groups were quite similar (19% vs. 14.4% and 15.1% vs. 12.9%, respectively). A Cox multivariate regression analysis including age, international staging system immunoglobulin isotype, and XPO5, KRT81 and TRBP miRSNPs showed that the SNP in XPO5 rs11077 was the only parameter to retain its prognostic impact on PFS and OS (\( P = 0.028 \) and \( P = 0.014 \), respectively). For historical reasons, there was only cytogenetic information in 41 patients. The distribution of poor prognostic alterations [t(4;14), t(14;16), and del17p] was not significantly different between the genotypic groups.
The miRSNP in KRT81 3'UTR affects protein translation in multiple myeloma cell lines

The analysis of the 3'UTR of KRT81 showed that the miRSNP is located in the miRNA-binding site for miR-17, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, and miR-519d (as predicted by TargetScan 5.1). The nucleotide change produces the loss of a nucleotide binding in the seed sequence region of these miRNAs as shown in Fig. 3A. To analyze the functional effect of this nucleotide change in the expression of KRT81, we constructed a vector containing the SNP or the WT genotype in the 3'UTR region of Renilla luciferase gene (WT/SNP-modified psiCheck2 vectors) and we transfected both vectors in 2 myeloma cell lines. A reduction by 9% and 25% in the Renilla luciferase activity analysis 48 hours after transfection was observed in KMM-1 (P = 0.14) and RPMI-8226 (P = 0.01), respectively, with the SNP-modified psiCheck2 vector (Fig. 3B). These results indicate that the presence of the polymorphisms enhances the binding of miRNAs to this sequence resulting in a reduction of protein levels.

The miRSNP in KRT81 3'UTR alters KRT81 levels in healthy lymphocytes

To verify the observed effect in protein reduction by SNP presence in the in vitro assays, we analyzed the mRNA and protein levels of KRT81 in lymphocytes from 50 healthy controls. No differences in mRNA levels were observed. Interestingly, we observed a genotype significant dose-dependent reduction in the KRT81 levels, where the WT genotype showed the higher levels and the SNP the lower levels (average reduction, 37%), with an intermediate value for heterozygous patients (average reduction, 15.48%; ANOVA, P = 0.033).

KRT81 reduction in myeloma cell lines are related to lower proliferation

Because we have observed that the presence of the SNP is related to lower protein production and moreover the patients harboring the homozygote SNP genotype had better prognosis, we wanted to study whether or not the reduction of KRT81 gene is affecting the proliferative rate in myeloma cell lines. Then, we transfected both myeloma cell lines with a KRT81-siRNA or with a control-siRNA and analyzed the proliferation using MTS at 24 hours of transfection. A reduction in the proliferation rate by 27.31% (P = 0.1) and 61.11% (P = 0.045) for KMM-1 and RPMI-8226, respectively, was observed in the KRT81-siRNA–transfected cells (Fig. 4).

The miRSNP in XPO5 3'UTR affects protein translation in multiple myeloma cell lines

The analysis of the 3'UTR region of XPO5 did not show any miRNA-binding site in the miRSNP region which can be affected by the nucleotide change (TargetScan 5.1). However, the nucleotide change produces the appearance of a binding site for miRNA hsa-miR-4763-5p (http://www.mirbase.org/search.shtml) as shown in Fig. 5A. To analyze the functional effect of this nucleotide change in the expression of XPO5, we have constructed a vector containing the SNP or the WT genotype in the 3'UTR region of Renilla luciferase gene (XPO5-WT/SNP–modified psiCheck2 vectors) and we have transfected both vectors in 2 myeloma cell lines. A reduction by 18% and 25% in the Renilla luciferase activity analysis 24 hours after transfection was observed in KMM-1 and RPMI-8226, respectively, with the SNP-modified psiCheck2 vector (Fig. 5B). This result showed that the presence of the polymorphisms allows the binding of new miRNAs to this sequence producing a significant reduction of XPO5 protein levels.

The miRSNP in XPO5 3'UTR alters XPO5 levels in healthy lymphocytes

To analyze whether the miRSNP in XPO5 is playing a role in prognosis by affecting the XPO5 levels, we analyzed the mRNA and protein levels of XPO5 in 50 healthy controls. Although no differences in mRNA levels were observed, the C/C genotype was associated with a 17.4% reduction of the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** A, schematic representation of how the miRSNP rs3660 located in the 3’UTR region of KRT81 gene is affecting the miRNA binding. B, Renilla/luciferase assay at 48 hours with the KRT81 WT or SNP-modified psiCheck2 vector transfected in KMM-1 and RPMI-8226 myeloma cell lines.
related protein, XPO5 \( [P = \text{not significant (NS)}] \), on peripheral blood lymphocytes.

**Discussion**

In the present study, we report for the first time that polymorphisms related to microRNAs (miRSNPs) have a prognostic impact in multiple myeloma. Two miRSNPs, KRT81 and XPO5, are involved in the prognosis after melphalan-based ASCT of patients with multiple myeloma.

Keratins have been recognized as regulators of cellular functions, including polarization, protein synthesis, membrane traffic, and signaling (16). To date, the only keratin for which a specific DNA variant has been associated with cancer predisposition is KRT5 in basal cell carcinoma (17). Notwithstanding the former, their expression levels have been described as prognostic markers in several epithelial tumors (16). SNP rs3660 is located in the 3' UTR of KRT81, a predicted binding site for several miRNAs such miR-17 and miR-93, among others (13), which has been previously observed to be upregulated in multiple myeloma (18).

Their functions include downregulation of expression of SOCS-1, a gene that plays a critical role as inhibitor of interleukin (IL)-6 growth signaling (18). In the present study, we have observed in 2 myeloma cell lines, through a Renilla luciferase experiment, that the presence of the SNP can affect the final protein levels, where the presence of the C allele in the 3' UTR facilitates the binding of the miRNAs producing a reduction in the protein levels. This observation is related to the loss of a G=C binding by the presence of the polymorphism. This alters hybridization process, affecting the minimum free energy (mfe). Using as example, KRT81-miR-17 binding, we can observe that the presence of the SNP reduces the mfe necessary for the hybridization from WT-mfe = −8.8 kcal/mol to SNP-mfe = −10.7 kcal/mol (as predicted by RNAHybrid: http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). When more negative the free energy of a structure, the more likely is formation of that structure. Then, the presence of the SNP facilitates the RNA–miRNA binding. Interestingly, this result is confirmed by protein analysis of KRT81 levels in lymphocytes from healthy controls, where we found significant differences in KRT81 levels according the genotype.

To further investigate the functional consequences of alteration of KRT81 levels in multiple myeloma, we analyzed proliferation levels after KRT81 degradation by siRNA, observing that the presence of the SNP was correlated to lower proliferation. Both lines were affected in a similar way, but with a different degree, by the silent mRNA for KRT81 and with the transfection with a vector to SNP or the WT genotype in the Renilla luciferase gene. Baseline

**Figure 4.** Proliferation rate analyzed by MTS at 24 hours of transfection of both myeloma cell lines with a KRT81-siRNA cell lines with a control-siRNA. OD, optical density.

**Figure 5.** A, schematic representation of the apparition of a binding site for miRNA hsa-miR-4763-5p when miRSNP rs11077 in XPOS appears. B, Renilla/luciferase assay at 48 hours with the XPOS WT or SNP-modified psiCheck2 vector transfected in KMM-1 and RPMI-8226 myeloma cell lines.
proliferation, more prominent in RPMI-8226 cells, could be a theoretical reason. We measured the mRNA level for KRT81 by RT-PCR in both cell lines. KRT81 was 10.2% less expressed in KMM-1 than in RPMI-8226 (P = NS). Thus, differential expression of this gene could also be involved in these findings. All these results allows us to speculate that the observed better OS in patients with multiple myeloma harboring the SNP could be explained by the reduction in the levels of KRT81 in this group of patients which are related with a lower proliferative phenotype of tumor cells. Moreover, recently in the whole genome sequencing of malignant plasma cells, mutations in some keratins (i.e., KRT31) have been described (19). These recent findings support the importance of keratin proteins in multiple myeloma. Validation in larger series is warranted, particularly when some association such as OS with KRT81 rs3360 would perhaps not be statistically significant if corrected P values for multiple comparisons would have been carried out.

XPO5 is found in the nuclear membrane and mediates the transport of proteins and other cargo between the nucleic and cytoplasmic compartments. One of these functions involves miRNA biosynthesis (20). The first precursor of miRNA, the primary miRNA, goes through nuclear maturation by RNAase DROSHA system, forming the pre-miRNA. XPO5 is then incharged to relocate this molecule in the cytoplasm, where the RNAase DICER complex finished the miRNA maturation process. In some tumor cells, particularly with microsatellite instability, a mutated and inactive XPO5 traps pre-miRNAs in the nucleus, resulting in a reduced miRNA processing, as well as a decreased miRNA target inhibition (21). Moreover, the restoration of XPO5 functions reverses the impaired export of pre-miRNAs and has tumor suppressor features. rs11077 is a polymorphism found in the 3’UTR region of the gene. It was first correlated with esophageal cancer risk (5). Of interest, this SNP has recently been associated with a trend toward a higher disease control rate in metastatic colon cancer (22). Our group has recently reported a correlation with time to progression in non–small cell lung cancer (14). However, the molecular mechanism by which these polymorphisms lead to improving the prognosis after ASCT is not yet understood. It could be possible that SNPs in the downstream region of XPO5 could be associated with altered expression levels of this protein and, consequently, with quantitative variations in mature miRNA levels in the cytoplasm (8). To prove this hypothesis, we have analyzed whether the expression of Renilla luciferase is affected by the genotype. Interestingly we observed a reduction in the Renilla levels in the vector containing the SNP variant. One possible explanation for this protein levels reduction with the SNP variant is the fact that the presence of the SNP allows the binding of miRNAs that has not previously predicted by bioinformatic methods (miRbase, TargetScan). Moreover, we observed a trend to a lower protein expression in healthy donor lymphocytes harboring the SNP genotype. Sample size and probably many involved heterogeneous factors could condition these levels and limit the statistical conclusions in this regard. Despite of a lack of difference between the response degrees after ASCT, potential variable plasma cell sensitivity to melphalan exposure cannot be excluded and further investigation in this regard is warranted. Interestingly, other members of exportin family have been observed altered in cancer. Thus, a recurrent mutation in other exportin (XPO1) has been described in patients with chronic lymphatic leukemia with unmutated immunoglobulins, a finding that supports the implications of this carries in hematologic malignancies (23).

In summary, this is the first report concerning miRSNPs involved in the miRNA network in hematologic malignancies. A polymorphism in the binding site for diverse miRNA clusters in KRT81, a relevant gene in the structural cytoplasm framework, was associated with prognosis in multiple myeloma. Moreover, an SNP in XPO5 was associated with a significantly longer PFS and OS in patients with multiple myeloma after ASCT. MiRSNPs emerged as new promising markers for disease progression in cancer and specifically in multiple myeloma.

Disclosure of Potential Conflicts of Interest

J Bladé received a grant support from Janssen-Cilag. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Fernández de Larrea, A. Navarro, M. Monzó, J. Bladé
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Fernández de Larrea, A. Navarro, N. Tovar, M.T. Cibeira, M. Rozman, J. Bladé, L. Rosiñol
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Fernández de Larrea, A. Navarro, R. Tejero, J. Bladé, T. Diaz, G. Ferrer
Writing, review, and/or revision of the manuscript: C. Fernández de Larrea, A. Navarro, M.T. Cibeira, L. Rosiñol, M. Rozman, M. Monzó, J. Bladé, N. Tovar, T. Diaz, R. Tejero, G. Ferrer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Fernández de Larrea
Study supervision: A. Navarro, M. Monzó, J. Bladé

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Carlos Fernández de Larrea, Alfons Navarro, Rut Tejero, et al.

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