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# miR-18b overexpression identifies mantle cell lymphoma patients with poor outcome and improves the MIPI-B prognosticator

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## **Key Points**

- miR-18b overexpression identifies patients with poor prognosis in two large prospective MCL cohorts treated with Rituximab-chemo and ASCT
- miR-18b overexpression adds prognostic information to the MIPI-B prognosticator

## **Abstract**

Recent studies show that mantle cell lymphoma (MCL) express aberrant miRNA profiles, however, the clinical effect of miRNA expression has not previously been examined and validated in prospective, large, homogenously treated cohorts. We analyzed diagnostic MCL samples from the Nordic MCL2 and MCL3 clinical trials, in which all patients had received Rituximab-high-dose cytarabin alternating with Rituximab-maxiCHOP, followed by BEAM and autologous stem cell support. We performed genome-wide miRNA microarray profiling of 74 diagnostic MCL samples from the MCL2 trial (screening cohort). Differentially expressed miRNAs were re-analyzed by qRT-PCR. Prognostic miRNAs were validated by qRT-PCR in diagnostic MCL samples from 94 patients of the independent MCL3 trial (validation cohort).

Three miRNAs (miR-18b, miR-92a, miR-378d) were significantly differentially expressed in patients who died from MCL in both the screening- and the validation cohort. MiR-18b was superior to miR-92a and miR-378d in predicting high risk. Thus, we generated a new MIPI-B-miR prognosticator, combining expression-levels of miR-18b with MIPI-B data. This prognosticator improved identification of high risk patients compared to MIPI-B with regard to cause-specific survival ( $P=0.015$ ), overall survival ( $P=0.006$ ) and progression-free survival ( $P<0.001$ ).

Transfection of two MCL cell lines with miR-18b decreased their proliferation rate without inducing apoptosis, suggesting miR-18b may render MCL cells resistant to chemotherapy by decelerating cell proliferation.

Thus, we conclude that overexpression of miR-18b identifies patients with poor prognosis in two large prospective MCL cohorts and adds prognostic information to MIPI-B. MiR-18b may reduce the proliferation rate of MCL cells as a mechanism of chemoresistance.

## Introduction

Mantle-Cell Lymphoma (MCL) is an aggressive B-cell lymphoma subtype characterized by the pathogenic hallmark, the t(11;14)(q13;q32) translocation, which leads to overexpression of the cell-cycle promoting protein Cyclin D1.<sup>1</sup> New treatment regimens, including CD20-antibodies, high dose chemotherapy and autologous stem cell support (ASCT), have improved survival exceptionally.<sup>2-4</sup> Despite this, long-term follow up data from the Nordic MCL2 trial show that 57% of younger MCL patients ( $\leq 65$  years) relapse during a 10-year period.<sup>5</sup> Risk-stratification is therefore of great importance in order to identify patients who are eligible for novel or alternative treatment regimens. The current prognostic models, the clinical MCL International Prognostic Index (MIPI),<sup>6</sup> and the biological MIPI (MIPI-B), which includes staining with the proliferation marker Ki-67, assign patients to high, intermediate, or low risk groups. This prognosticator has been investigated in several cohorts<sup>4,7-11</sup> and was useful in most.<sup>7-10</sup> The MIPI-B does not, however, identify specific biological pathways designating aggressive subtypes of MCL.

MicroRNAs (miRNAs) are a class of non-coding RNAs that inhibit translation of specific messenger RNAs (mRNAs), and can function as both tumor suppressor miRNAs and oncogenic miRNAs depending on the cellular context.<sup>12</sup> Because of their small size (19-22 nucleotides), miRNAs are well preserved and can be successfully examined in formalin-fixed paraffin-embedded (FFPE) tissue, showing expression levels that correlate with those obtained in cryopreserved tissue.<sup>13</sup> We have previously purified miRNAs from FFPE tissue in cutaneous T-cell lymphoma<sup>14</sup> and diffuse large B-cell lymphoma,<sup>15</sup> and obtained reproducible miRNA levels in samples stored for up to 30 years.

Over the last five years, several studies have shown that miRNAs delineate aberrant molecular pathways and predict survival in MCL patients.<sup>16,17</sup> Furthermore, the knockdown of specific miRNAs in two MCL-mouse models has demonstrated efficacy in inhibiting lymphoma growth.<sup>18,19</sup> However, there has been only modest consistency between the clinical studies, and at

this point, miRNA expression in MCL has not yet been examined in large, prospective, uniformly treated patient cohorts.

To identify aberrant biological pathways and find prognostic biomarkers, we examined miRNA expression levels in 168 MCL patients from the almost identical Nordic MCL2 and MCL3 trial cohorts according to REMARK guidelines.<sup>20</sup> All patients had been treated with high-dose immunochemotherapy and ASCT, and had long-term follow-up. We initially analyzed the genome-wide miRNA expression-profile of diagnostic patient samples in the Nordic MCL2 cohort and linked these findings with comprehensive clinical follow-up data. Validation of differentially expressed miRNAs was performed in samples from patients in the Nordic MCL3 cohort to determine the prognostic value of miRNA expression in MCL patients. Finally, the functional implications of aberrant miRNA expression were studied in MCL cell lines.

## **Methods**

Figure 1 provides a flowchart summarizing the experimental procedures.

### ***Study population***

All patients included in this study have been enrolled in two similar prospective trial cohorts; The 2<sup>nd</sup> Nordic Lymphoma Group MCL Trial cohort (MCL2), or The 3<sup>rd</sup> Nordic Lymphoma Group MCL Trial cohort (MCL3). The study designs of the MCL2 and MCL3 trials have been published previously.<sup>2,21</sup> Briefly, both cohorts consisted of newly diagnosed, previously untreated, stage II-IV MCL patients below the age of 66 years. All diagnostic specimens were centrally reviewed by at least one member of the central Nordic MCL Pathology board and the diagnosis confirmed according to World health Organization (WHO) criteria.<sup>22</sup> All patients were followed with a complete work-up including clinical history, physical examination, blood and bone-marrow examination, and CT or PET-CT scans on study entry and subsequently every 6 months for 5 years. After 5 years, patients were followed with annual clinical assessment, standard blood tests, and minimal residual disease (MRD) analysis.<sup>23</sup>

Both cohorts received the same treatment regimens consisting of rituximab(R)-maxi-CHOP alternating with R-high-dose Ara-C, followed by high-dose BEAM or BEAC and ASCT. The only difference between the two regimens was that MCL3 patients with unconfirmed complete remission (CRu) or partial remission (PR) received <sup>90</sup>Y-Ibritumomab-Tiuxetan (Zevalin) in addition to BEAM or BEAC before ASCT. The addition of Zevalin did not have any impact on treatment outcome or adverse events, and overall survival, event-free survival, and time to progression were similar in the two cohorts.<sup>21</sup> In both cohorts, a fraction of these patients also received pre-emptive Rituximab at molecular relapse during follow up.<sup>23</sup>

### ***Patient samples***

A total of 172 FFPE preserved diagnostic MCL biopsies from 172 MCL patients from both cohorts (76 MCL2 and 96 MCL3, respectively) were retrieved from the respective Nordic MCL pathology board. Four patients were excluded due to poor quality of the RNA extracted from the MCL specimens. All included patient samples had confirmed overexpression of cyclin D1 or carried the t(11;14)(q13;q32) translocation. Ki-67 expression was evaluated by immunohistochemistry in 145 patient samples, and a marker for MRD<sup>23</sup> was available in 96 of these.

The clinical trials (MCL2: ISRCTN 8786 6680, MCL3: NCT 00514475) were conducted in accordance with the Declaration of Helsinki, and approved by the relevant ethic committees of all Nordic countries. Informed consent was obtained from all participants.

### ***RNA extraction from FFPE-tissue***

Total RNA from all samples was isolated from four 10µm tissue sections using the RecoverAll total nucleic acid isolation kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's guidelines. The samples were incubated in Xylene at 50°C to remove paraffin, followed by ethanol wash. Proteins were degraded by digestion buffer and protease at 50/80°C. Subsequently, isolation buffer and ethanol were added, samples were bound to a spin-column, and DNA was degraded by DNase treatment. The filter was washed and total RNA was eluted in a 60 µL elution solution. Total RNA quantity (OD 260 nm) and quality (260/280 ratio) were measured by an ND-1000 spectrophotometer (Thermo Scientific, Delaware, USA).



### ***miRNA microarray analysis***

For miRNA microarray profiling, 500 ng of total RNA from samples or reference (AM6000; common RNA pool for identifying a maximum number of expressed miRNAs in patient samples, Ambion, Carlsbad, CA, USA) was labeled with Hy3 and Hy5 fluorescent dyes, respectively, using the miRCURY LNA array power labeling kit (Exiqon A/S, Vedbæk, Denmark). Samples with RNA concentrations lower than 160 ng/μl were vacuum-centrifuged (speedvac) to obtain higher concentrations. All samples were labeled the same day with the same master mix to minimize technical variation.

The labeled samples were hybridized to miRCURY LNA 7<sup>th</sup> generation arrays (Manual version 2.2; Exiqon A/S, Vedbæk, Denmark), containing capture probes targeting all human miRNAs registered in miRBASE release 18.0 (1896 human miRNAs). The hybridization was performed overnight at 56°C according to the manufacturer's specifications using an HS4800 hybridization station (Tecan, Männendorf, Switzerland). Since it was not possible to hybridize all arrays at the same time point, samples were randomly split into six batches to minimize day-to-day variation in the hybridization process, and analyzed over six consecutive days. After hybridization, the microarray slides were scanned in an ozone free environment in order to prevent potential bleaching of the fluorescent Hy5 dye. Scanning was done using a G2565BA Microarray Scanner system (Agilent Technologies, Santa Clara, CA, USA) at 10-μm resolution, and the resulting TIFF images were analyzed using the GenePix 6.1 software on standard settings.

Microarray data can be found in the Gene Expression Omnibus database

(<http://www.ncbi.nlm.gov/geo/>) with accession GSEXXXX.

### ***Microarray data processing***

Processed microarray image data (Genepix median spot intensities) were read into the R statistical environment and preprocessed using the LIMMA package.<sup>24</sup> The arrays were background corrected using the 'normexp' method with an offset of 10 to account for spatial biases as suggested by Ritchie et al. (2007),<sup>25</sup> and between-array variance was eliminated by quantile normalization.<sup>26,27</sup> Expression profiles and clinical variables were subjected principal component analysis (singular-value decomposition) to assess whether data were affected by unexpected confounding effects. A differential expression analysis of the foreground intensities was conducted for the cause-specific death contrast. Empirical Bayes moderated statistics were assessed and adjusted for multiple testing using the Benjamini-Hochberg method.

### ***Quality assessment of patient samples***

Principal components analysis of miRNA microarray data and sample related parameters (anatomical site of origin of MCL-biopsy, country, batch, speedvac-procedure, cytology or growth pattern) confirmed that none of these factors had any influence on the quality of the microarray data (data not shown).

### ***miRNA qRT-PCR***

Ten ng of RNA were reverse transcribed in 10µL reactions using miRCURY LNA Universal RT miRNA PCR, polyadenylation, and cDNA synthesis kit (Exiqon A/S). cDNA was diluted 40 times and assayed in duplicate 10µL PCR reactions according to protocol. Negative controls excluding template from the reverse transcription reaction were performed and profiled in parallel. The qRT-PCR reactions were performed on a LightCycler II instrument (Roche, Basel, Switzerland) in 96-

and 384-well plates. The amplification curves were analyzed using Roche LightCycler version 1.5.1.62 software, both for determination of crossing point (Cp, by the second derivative method) and for melting curve analysis. All assays were inspected for distinct and uniform melting curves to verify that the correct amplicon was amplified in all samples. Furthermore, samples were only included in the data analysis if their Cp-value was 3 less than the negative control, and with Cp < 39. Samples that did not pass these criteria were omitted from further analysis. To eliminate bias of miRNA concentrations due to differences in initial RNA concentration, data were normalized using averaging windows (LOESS method). MiR-500a-5p, miR-335-3p and miR-423-5p had lowest variance among expressed miRNAs on the microarray, which were confirmed by qPCR, and were selected as reference miRNAs.

### ***Statistics and response criteria***

Differences in baseline characteristics between the two cohorts were calculated using the Pearson chi-squared test and Mann-Whitney non-parametric test. Outcome-related variables were assessed with Kaplan-Meier analyses and compared using the log-rank test. The prognostic information of the miRNAs was evaluated using the minimal-redundancy-maximal-relevance (mRMR) criterion.<sup>26,27</sup> Comparison of the MIPI-B prognosticator and the MIPI-B-miR prognosticator was performed with respect to the prediction of high risk-groups. Statistical analyses were performed using SPSS version 20.0.0 (IBM, NY) and R version 2.1.4 ([www.r-project.org](http://www.r-project.org)). Two-sided tests with a significance level of 5% were used for all analyses.

The prospective nature of the two MCL trials enabled us to use cause-specific survival (CSS) as primary endpoint according to response criteria of the International Workshop.<sup>28</sup> Progression-free survival (PFS) and overall survival (OS) were used as secondary endpoints. Multivariate Cox regression analysis was performed with backward selection to assess prognostic factors.

### ***Cell culture***

The MCL cell line, Z-138, was obtained from ATCC whereas Jeko-1 was provided by the European MCL Network. The cells were maintained as suspension cultures in either RPMI (Jeko-1) or IMDM (Z-138) supplemented with 20% heat inactivated fetal calf serum (FCS), 10 U/mL penicillin, and 10 µg/mL streptomycin. All tissue culture reagents were purchased from Life Technologies. The cells were maintained in a humidified chamber with 5% CO<sub>2</sub> at 37°C.

### ***Electroporation***

Jeko-1 and Z-138 cells were transfected in a Nucleofector II device (Lonza) using program X-001. Briefly,  $5 \times 10^6$  cells were resuspended in 100 µl Solution V (Lonza) containing 1 µM of either miR-18b mirVana miRNA mimic (Life Technologies) or a scrambled negative control mirVana miRNA mimic (Life Technologies). Immediately after electroporation, the cells were transferred to pre-heated complete medium and incubated at 37°C until further analysis by Flow Cytometry (FACScalibur, Becton Dickinson) or XTT assay.

### ***Apoptosis and cell proliferation/viability assays***

Apoptosis was assessed using the Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Briefly, 24 hours after transfection  $1 \times 10^6$  cells were incubated with 5 µl Annexin V-PE and 5µl 7-AAD in 100 µl of  $1 \times$  Annexin V-binding buffer at room temperature. After incubating for 15 min, 400 µl of  $1 \times$  binding buffer was added, and the apoptotic cells were then analyzed by Flow Cytometry.

To study cell proliferation, Jeko-1 and Z-138 cells were seeded in 96-well plates immediately after transfection at a density of  $5-10 \times 10^4$  cells/well in 50 or 100 µl complete medium, respectively. Cell number was determined 0-7 days after transfection by adding 25 or 50 µl 2,3-bis[2-methoxy- 4-nitro-sulfohenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT; Roche

Diagnostics) reagent to each well and measuring the absorbance at 450 nm (reference 650 nm) after 3h of incubation at 37°C.

## Results

### *Clinical characteristics of the study cohorts*

Baseline characteristics of MCL2 and MCL3 patients were similar with respect to gender, initial duration of response, WHO performance status, white blood cell count (WBC), lactate dehydrogenase (LDH), age, Ki67 expression and MIPI score, but differed significantly with regard to cytological variant. Follow-up was complete for all patients of both cohorts, with a median follow-up time of 6.4 years and 3.7 years, respectively (supplemental Table 1).

### *miRNA microarray screening of the MCL2 cohort reveals 17 differentially expressed miRNAs in patients who die from MCL*

We initially assessed the global miRNA profile of 74 diagnostic samples from patients of the MCL2 cohort by miRNA microarray. To identify prognostic miRNAs in MCL, each microarray was subjected to a statistical hypothesis test (see Methods) with regard to the CSS. This endpoint was chosen to evaluate the impact of the specific biological (miRNA) aberrations, and was possible because of the extensive follow-up in the two clinical trials. Seventeen miRNAs were found to be differentially expressed (adjusted p-values < 0.05; see Table 1), with 14 miRNAs being up-regulated and 3 miRNAs being down-regulated in patients who died from MCL. A heatmap of the differentially expressed miRNAs is shown in Figure 2.

### *qRT-PCR confirms miRNA microarray findings in the MCL2 cohort*

To confirm the microarray findings we chose 26 miRNAs to be analyzed by qRT-PCR in the same 74 MCL2 patient samples: Seventeen miRNAs were selected on the basis of adjusted p-values < 0.05 on the microarray (Table 1), and two miRNAs were selected on the basis of combined adjusted p-values < 0.1 and differential expression in previous MCL studies (miR-146a-5p, miR-92a-3p).<sup>18,29</sup> Four additional miRNAs (miR-29a, miR-29b, miR-29c and miR-20b), also known from the

literature,<sup>30,31</sup> but not differentially expressed in our microarray analysis, were also chosen for qRT-PCR analysis in MCL2. Three miRNAs were selected as reference miRNAs on the basis of their low variance of expression across all microarrays (supplemental Figure 1).

With regard to CSS, seven of the miRNAs selected for qRT-PCR analysis (miR-4417, miR-18b-5p, miR-3687, miR-378d, miR-185-5p, miR-144-3p, and miR-92a-3p) were differentially expressed thus confirming the microarray findings in the MCL2 cohort (Table 1). Log fold changes tended to be higher in the qRT-PCR analysis compared to the microarray, and the adjusted p-values were correspondingly lower by qRT-PCR than by microarray.

### ***High expression of miR-18b predicts poor prognosis in MCL***

Previous studies have identified various miRNAs that predict outcome in heterogeneous cohorts of MCL patients,<sup>16,17</sup> however, the prognostic value of miRNA profiles in similarly treated cohorts of MCL patients remains to be demonstrated. To evaluate the prognostic performance of the differentially expressed miRNAs we performed mRMR analysis and a leave-one-out cross-validation using linear discriminant analysis (LDA). The maximum enhancement of the prognostic accuracy of MIPI-B was achieved by miR-18b expression (supplemental Figure 2), and, in addition, a univariate analysis revealed that miR-18b overexpression was significantly correlated with poor survival in MCL2 ( $P < 0.001$ , log-rank) (supplemental Figure 3).

### ***Validation in the independent MCL3 cohort***

In order to validate findings from the MCL2 cohort, we analyzed the confirmed differentially expressed miRNAs in the independent, but similarly treated, MCL3 cohort by qRT-PCR. Here we were able to validate miR-18b, miR-378d and miR-92a as being significantly overexpressed (adjusted  $P < 0.05$ ) in patients who died from MCL (Table 1). Both miR-18b and miR-92a have previously been associated with aggressive subtypes of MCL, whereas miR-378d is a novel prognostic factor for survival in MCL.

### ***MIPI-B-miR: Addition of miR-18b expression improves the prognostic value of MIPI-B***

Several studies, including the Nordic MCL2 trial,<sup>7</sup> have confirmed the MIPI-B score as the best performing prognosticator of MCL outcome. In order to assess the prognostic value of miRNA expression, we incorporated the MIPI-B components with expression data of miR-18b. Based on the MCL2 screening cohort, we developed a novel prognostic index using LDA of miR-18b expression levels and MIPI-B value to assign patients to high, intermediate or low risk. This new prognostic index, MIPI-B-miR, was defined as:  $\omega' = \omega + cx$ , where  $\omega$  is the MIPI-B value,  $x$  is the log-fold-change of miR-18b, and  $c$  is a regression parameter ( $c=0.58317$ ). The low risk was defined as  $\omega' < 5.75$ , high risk was defined as  $\omega' > 7.49$  and intermediate risk is implied when MIPI-B-miR values fall between these thresholds (supplemental text).

MIPI-B-miR had a significantly better separation of patients in low, intermediate and high risk groups compared to the standard MIPI-B regarding overall survival (OS) and progression-free survival (PFS), and was borderline significant with regard to CSS (Figure 3A-C). In particular, the MIPI-B-miR reduced the number of high-risk patients by two thirds, and identified a group of patients with an exceedingly poor outcome compared to that identified by the MIPI-B high-risk index.

To confirm these findings we blindly tested the MIPI-B-miR prognosticator on the independent MCL3 validation cohort. The MIPI-B-miR also improved the separation of risk groups in the validation cohort regarding CSS, OS and PFS (Figure 4 A-C), e.g. MIPI-B-miR achieved significant prognostic value for OS ( $P < 0.001$ ), while MIPI-B does not ( $P = 0.143$ ). Compared to MIPI-B, MIPI-B-miR showed a trend of improved identification of high risk patients (CSS:  $P = 0.101$ , OS:  $P = 0.101$  and PFS:  $P = 0.097$ ), despite the shorter observation time in this cohort.

Based on the improved prognostication by MIPI-B-miR, we next applied this index to the entire patient material (MCL2 and MCL3). In the combined analysis MIPI-B-miR was superior to the standard MIPI-B at identifying high risk patients with respect to CSS, OS and PFS (Figure 5 A-C and supplemental Figure 4). In the subset of patients with a known MRD marker, MIPI-B-miR



predicted a high risk of molecular relapse ( $P < 0.001$ ), while MIPI-B did not ( $P = 0.322$ ; supplemental Figure 5). Importantly, MIPI-B-miR was still significantly superior to MIPI-B at predicting CSS ( $P = 0.034$ ), OS ( $P = 0.034$ ) and PFS ( $P = 0.006$ ) when patients receiving pre-emptive Rituximab were excluded from the analysis of the combined cohort (MCL2 and MCL3) (supplemental Figure 6). Finally, a multivariate analysis of the combined MCL2 and MCL3 cohorts confirmed miR-18b as an independent prognostic factor for OS (supplemental Table 3).

### ***MiR-18b function***

To investigate the possible functional consequences of miR-18b overexpression, two MCL cell lines, Z-138 and Jeko-1, were selected due to their high transfection efficiency. Both cell lines express miR-18b at intermediate levels compared with primary MCLs expressing high or low levels of miR-18b (Figure 6 A). Z-138 and Jeko-1 cells transfected with miR-18b mimic display significantly lower viability scores compared to cells transfected with scrambled miRNA as measured by XTT assay ( $P = 0.05$  and  $P = 0.005$ , respectively) (Figure 6 B). The low cell viability observed upon miR-18b overexpression was not associated with the induction of apoptosis, as no increase in the early apoptotic cell population was detected when miR-18b transfected cells were compared to scrambled miRNA transfected cells (Figure 6 C). However, when comparing the proliferation rate between cells transfected with miR-18b or scrambled miRNA, miR-18b was found to markedly inhibit the proliferative ability of both Z-138 and Jeko-1 cells over an 8-day test period although this was most pronounced in Jeko-1 cells (Figure 6 D). Overexpression of miR-18b in Jeko-1 and Z-138 cells did not alter their cell cycle profile, when compared to scrambled miRNA transfected cells (data not shown), indicating miR-18b likely inhibits cell cycle progression at multiple points.

## Discussion

This study of 168 patients provides the most comprehensive study of miRNA expression profiles in MCL to date. Moreover, it is the first study to prospectively follow and analyze miRNA expression in a large, homogeneously treated cohort of MCL patients. The detailed trial data also enabled us to study CSS, excluding treatment-related deaths or deaths from other causes. By analyzing the expression of 1896 human miRNAs by microarray in patients from the MCL2 cohort, we identified 17 miRNAs that were significantly up- or down-regulated in patients who died from MCL. qRT-PCR analysis confirmed that seven of these were up-regulated. Subsequent validation in the independent MCL3 cohort identified an aggressive MCL subtype, biologically characterized by aberrant overexpression of miR-18b, miR-92a and miR-378d. These miRNAs, and miR-18b in particular, signify MCL patients that are incurable by the current standard therapy R-maxi-CHOP/R-high-dose Ara-C and BEAM/-C and ASCT.

The prognostic impact of miRNAs have previously been studied in MCL, but mainly in smaller, heterogeneous cohorts, treated by variant regimens<sup>16,17</sup> These studies have suggested a number of prognostic miRNAs including miR-18b,<sup>29</sup> and the entire miR-17~92a cluster. However, no other study identified miR-92a as a key component. Here, we confirm that these miRNAs are valid prognostic markers in our large cohort of homogeneously treated MCL patients. Moreover, we observed that other previously published prognostic miRNAs (miR-146a, miR-20b, miR-29a, miR-29c)<sup>29-31</sup> displayed trends in our data that agreed with earlier findings, but were not statistically significant in this study.

From a genomic perspective, miR-18b and miR-92a are located in two paralogue clusters (the miR-106a~363 cluster on chromosome X and the miR-17~92a cluster on chromosome 13, respectively) with shared evolutionary origin.<sup>32</sup> In particular, overexpression of the miR-17~92a cluster has been widely studied, and has been implicated in both benign and malignant lymphoproliferative diseases,<sup>33</sup> whereas the functions of the miRNAs in the miR-106a~363 cluster, which share

numerous seed sequences with the 17~92a cluster miRNAs, are less well explored.<sup>34</sup>

Overexpression of miR-378d has been associated with poor prognosis in acute myeloid leukemia,<sup>35</sup> and with brain metastasis in non-small cell lung cancer,<sup>36</sup> but has to our knowledge not been implicated in lymphoid malignancies.

Although aberrantly expressed miRNAs have been suggested as prognostic markers for survival in MCL, no study has yet identified miRNAs that could improve upon the state-of-the-art prognosticator, the MIPI-B.<sup>16,17</sup> In the current study of two similar MCL trial cohorts, we find that the miR-18b expression level significantly adds prognostic information to the MIPI-B approach. By generating a new prognosticator, the MIPI-B-miR, we were able to predict survival of MCL patients after high-dose immunochemotherapy and ASCT and improve the gold standard prognosticator MIPI-B. Since the MIPI-B-miR was validated in a similarly treated cohort, and was also found to be significant in the whole test group, we believe this prognosticator may also have prognostic power in other cohorts of younger MCL patients treated by current standards (R-chemo-ASCT). In addition, MIPI-B-miR was superior to MIPI-B in predicting molecular relapse. Because exclusion of cases treated with pre-emptive Rituximab did not change the overall picture, the MIPI-B-miR may also be valid in settings without MRD surveillance and pre-emptive therapy. Future investigations using MIPI-B-miR will be required to demonstrate its validity in differently treated and elderly cohorts of MCL patients. In this study, quantification of miRNAs was performed by standard primer-based qRT-PCR on RNA extracted from FFPE preserved MCL-specimens, which are easily storable and accessible, emphasizing the feasibility and the robustness of the method.

Beyond their value as prognostic markers, aberrantly expressed miRNAs may be therapeutic targets in patients,<sup>37</sup> potentially enabling new therapies in the poor prognosis group. Unfortunately, the direct functions of these upregulated miRNAs in MCL are largely unknown. In particular divergent roles of miR-18b in carcinogenesis have been reported and complicate therapeutic approaches. For example, low expression of miR-18b has been shown to predict melanoma progression and short survival,<sup>38</sup> whereas in hepatocellular carcinoma, high miR-18b

expression correlates with low survival rate.<sup>39</sup> This is not surprising considering that different functions have been previously attributed to individual miRNAs in different cellular settings,<sup>12</sup> and interestingly, high expression of miR-18b is characteristic of human embryonic stem cells.<sup>40</sup> We have investigated miR-18b function in two MCL cell lines and observed that miR-18b overexpression significantly down regulated MCL cell proliferation. This observation supports that miR-18b adds prognostic information to MIPI-B by identifying an additional group of patients with poor prognosis, not identified by MIPI-B, which includes the proliferation marker Ki-67. The regimens used in the Nordic MCL2 and MCL3 trials comprise several cell cycle specific drugs including cytarabine, which will only target proliferating cells. Our *in vitro* studies clearly show an association between high miR-18b expression and low proliferation rate. We therefore suggest that high miR-18b expression may render MCL cells resistant to the MCL2/3 therapeutic regimens leading to poor prognosis. Inhibition of cell proliferation by miRNAs has previously been shown to play a pivotal role in chemoresistance, e.g. in osteosarcoma and colon cancer cells where both miR-140 and miR-215 cause chemoresistance by reducing the proliferation rate.<sup>41,42</sup> Since high expression of miR-18b is also typical of undifferentiated cells and embryonal carcinoma cells,<sup>40,43</sup> it is tempting to speculate if miR-18b expression can induce stemness in MCL, which is a subject for further studies.

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**Authorship and Conflict-of-Interest Statements**

Conceived and designed the experiments: SH, UR, RZ, JC, CHG, KG. Performed the experiments: SH, UR, RZ, LBP, AP. Analyzed the data: SH, UR, RZ, JC, CG, CTW, PB, KG. Contributed with patient material/reagents/materials/analysis tools: SE, AK, MJ, AL, RR, ME, CS, MK, JD, EC, PB, CHG, KG. Wrote the paper: SH, UR, RZ, JC, CG, CTW, CHG, KG. All authors read and approved the final manuscript.

The authors declare no competing conflicts of interest.

## References

1. Vaandrager JW, Schuurin E, Zwikstra E, et al. Direct visualization of dispersed 11q13 chromosomal translocations in mantle cell lymphoma by multicolor DNA fiber fluorescence in situ hybridization. *Blood*. 1996;88(4):1177–82.
2. Geisler CH, Kolstad A, Laurell A, et al. Long-term progression-free survival of mantle cell lymphoma after intensive front-line immunochemotherapy with in vivo-purged stem cell rescue: a nonrandomized phase 2 multicenter study by the Nordic Lymphoma Group. *Blood*. 2008;112(7):2687–93.
3. Damon LE, Johnson JL, Niedzwiecki D, et al. Immunochemotherapy and autologous stem-cell transplantation for untreated patients with mantle-cell lymphoma: CALGB 59909. *J. Clin. Oncol.* 2009;27(36):6101–8.
4. Van 't Veer MB, de Jong D, MacKenzie M, et al. High-dose Ara-C and beam with autograft rescue in R-CHOP responsive mantle cell lymphoma patients. *Br. J. Haematol.* 2009;144(4):524–30.
5. Geisler CH, Kolstad A, Laurell A, et al. Nordic MCL2 trial update: six-year follow-up after intensive immunochemotherapy for untreated mantle cell lymphoma followed by BEAM or BEAC + autologous stem-cell support: still very long survival but late relapses do occur. *Br. J. Haematol.* 2012;158(3):355–62.
6. Hoster E, Klapper W, Hermine O, et al. Confirmation of the mantle-cell lymphoma international prognostic index in randomized trials of the European mantle-cell lymphoma network. *J. Clin. Oncol.* 2014;32(13):1338–46.
7. Geisler CH, Kolstad A, Laurell A, et al. The Mantle Cell Lymphoma International Prognostic Index (MIPI) is superior to the International Prognostic Index (IPI) in predicting survival following intensive first-line immunochemotherapy and autologous stem cell transplantation (ASCT). *Blood*. 2010;115(8):1530–3.
8. Budde LE, Guthrie KA, Till BG, et al. Mantle cell lymphoma international prognostic index but not pretransplantation induction regimen predicts survival for patients with mantle-cell lymphoma receiving high-dose therapy and autologous stem-cell transplantation. *J. Clin. Oncol.* 2011;29(22):3023–9.
9. Rätty R, Honkanen T, Jantunen E, et al. Prolonged immunochemotherapy with rituximab, cytarabine and fludarabine added to cyclophosphamide, doxorubicin, vincristine and prednisolone and followed by rituximab maintenance in untreated elderly patients with mantle cell lymphoma: a prospective study. *Leuk. Lymphoma*. 2012;53(10):1920–8.
10. Todorovic M, Balint B, Andjelic B, et al. Outcome prediction of advanced mantle cell lymphoma by international prognostic index versus different mantle cell lymphoma indexes: one institution study. *Med. Oncol.* 2012;29(3):2212–9.
11. Shah JJ, Fayad L, Romaguera J. Mantle Cell International Prognostic Index (MIPI) not prognostic after R-hyper-CVAD. *Blood*. 2008;112(6):2583; author reply 2583–4.
12. Kasinski AL, Slack FJ. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat. Rev. Cancer*. 2011;11(12):849–864.

13. Culpin RE, Sieniawski M, Proctor SJ, Menon G, Mainou-Fowler T. MicroRNAs are suitable for assessment as biomarkers from formalin-fixed paraffin-embedded tissue, and miR-24 represents an appropriate reference microRNA for diffuse large B-cell lymphoma studies. *J. Clin. Pathol.* 2013;66(3):249–52.
14. Ralfkiaer U, Hagedorn PH, Bangsgaard N, et al. Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL). *Blood.* 2011;118(22):5891–900.
15. Hother C, Rasmussen PK, Joshi T, et al. MicroRNA Profiling in Ocular Adnexal Lymphoma: A Role for MYC and NFKB1 Mediated Dysregulation of MicroRNA Expression in Aggressive Disease. *Invest. Ophthalmol. Vis. Sci.* 2013;54(8):5169–75.
16. Husby S, Geisler C, Grønbæk K. MicroRNAs in mantle cell lymphoma. *Leuk. Lymphoma.* 2013;54(9):1867–75.
17. Goswami RS, Atenafu EG, Xuan Y, et al. MicroRNA signature obtained from the comparison of aggressive with indolent non-Hodgkin lymphomas: potential prognostic value in mantle-cell lymphoma. *J. Clin. Oncol.* 2013;31(23):2903–11.
18. Rao E, Jiang C, Ji M, et al. The miRNA-17~92 cluster mediates chemoresistance and enhances tumor growth in mantle cell lymphoma via PI3K/AKT pathway activation. *Leukemia.* 2011;(November):1–9.
19. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature.* 2010;467(7311):86–90.
20. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies. *J. Clin. Oncol.* 2005;23(36):9067–72.
21. Kolstad A, Laurell A, Jerkeman M, et al. Nordic MCL3 study: 90Y-ibritumomab-tiuxetan added to BEAM/C in non-CR patients before transplant in mantle cell lymphoma. *Blood.* 2014;123(19):2953–9.
22. Swerdlow SH, Campo E SM, Lymphoma. M-HH-KM cell, In: Swerdlow SH, Campo E H, NL, et al. E. Mantle cell lymphoma. *WHO Classif. tumours Haematop. lymphoid tissues.* 2008;229–32.
23. Andersen NS, Pedersen LB, Laurell A, et al. Pre-emptive treatment with rituximab of molecular relapse after autologous stem cell transplantation in mantle cell lymphoma. *J. Clin. Oncol.* 2009;27(26):4365–70.
24. Smyth GK. Limma: linear models for microarray data. *Bioinforma. Comput. Biol. Solut. using R Bioconductor.* 2005;397–420.
25. Ritchie ME, Silver J, Oshlack A, et al. A comparison of background correction methods for two-colour microarrays. *Bioinformatics.* 2007;23(20):2700–7.
26. Peng H, Long F, Ding C. Feature selection based on mutual information: criteria of max-dependency, max-relevance, and min-redundancy. *IEEE Trans. Pattern Anal. Mach. Intell.* 2005;27(8):1226–38.

27. Ding C, Peng H. Minimum redundancy feature selection from microarray gene expression data. *J. Bioinform. Comput. Biol.* 2005;3(2):185–205.
28. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J. Clin. Oncol.* 1999;17(4):1244.
29. Iqbal J, Shen Y, Liu Y, et al. Genome-wide miRNA profiling of mantle cell lymphoma reveals a distinct subgroup with poor prognosis. *Blood.* 2012;119(21):4939–4948.
30. Zhao J-J, Lin J, Lwin T, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood.* 2010;115(13):2630–9.
31. Di Lisio L, Gómez-López G, Sánchez-Beato M, et al. Mantle cell lymphoma: transcriptional regulation by microRNAs. *Leukemia.* 2010;24(7):1335–42.
32. Tanzer A, Stadler PF. Molecular evolution of a microRNA cluster. *J. Mol. Biol.* 2004;339(2):327–35.
33. Xiao C, Srinivasan L, Calado DP, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat. Immunol.* 2008;9(4):405–14.
34. Concepcion CP, Bonetti C, Ventura A. The MicroRNA-17-92 Family of MicroRNA Clusters in Development and Disease. *Cancer J.* 2012;18(3):262–7.
35. Qian J, Lin J, Qian W, et al. Overexpression of miR-378 is frequent and may affect treatment outcomes in patients with acute myeloid leukemia. *Leuk. Res.* 2013;37(7):765–8.
36. Chen L, Xu S, Xu H, et al. MicroRNA-378 is associated with non-small cell lung cancer brain metastasis by promoting cell migration, invasion and tumor angiogenesis. *Med. Oncol.* 2012;29(3):1673–80.
37. Janssen HLA, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* 2013;368(18):1685–94.
38. Dar AA, Majid S, Rittsteuer C, et al. The role of miR-18b in MDM2-p53 pathway signaling and melanoma progression. *J. Natl. Cancer Inst.* 2013;105(6):433–42.
39. Murakami Y, Tamori A, Itami S, et al. The expression level of miR-18b in hepatocellular carcinoma is associated with the grade of malignancy and prognosis. *BMC Cancer.* 2013;13(1):99.
40. Bar M, Wyman SK, Fritz BR, et al. MicroRNA discovery and profiling in human embryonic stem cells by deep sequencing of small RNA libraries. *Stem Cells.* 2008;26(10):2496–505.
41. Song B, Wang Y, Xi Y, et al. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. *Oncogene.* 2009;28(46):4065–74.
42. Song B, Wang Y, Titmus M a, et al. Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol. Cancer.* 2010;9:96.



43. Kushwaha R, Thodima V, Tomishima MJ, Bosl GJ, Chaganti RSK. miR-18b and miR-518b Target FOXN1 during epithelial lineage differentiation in pluripotent cells. *Stem Cells Dev.* 2014;23(10):1149–56.

## Tables

**Table 1**

**Significantly, differentially expressed miRNAs according to cause-specific survival**

miRNA	MCL2 (screening)		MCL3 (validation)
	Microarray adj. p-value	qPCR adj. p-value	qPCR adj. p-value
hsa-miR-124-5p	0,0002	-	-
hsa-miR-4417	0,0002	0,02	0,2309
<b>hsa-miR-18b-5p</b>	<b>0,0004</b>	<b>0,02</b>	<b>0,0002</b>
hsa-miR-3687	0,0046	0,01	0,1722
hsa-miR-519d	0,0046	-	-
<b>hsa-miR-378d</b>	<b>0,0092</b>	<b>0,02</b>	<b>0,0137</b>
hsa-miR-144-3p	0,0173	0,03	0,142
hsa-miR-486-5p	0,0173	0,05	0,0644
hsa-miR-1915-5p	0,0176	-	-
hsa-miR-665	0,0259	-	-
hsa-miR-185-5p	0,0308	0,04	0,059
<i>hsa-miR-4694-5p</i>	<i>0,0405</i>	-	-
<i>hsa-miR-5003-5p</i>	<i>0,0427</i>	-	-
hsa-miR-301a-3p	0,0439	0,43	-
hsa-miR-3182	0,0439	-	-
<i>hsa-miR-3591-3p</i>	<i>0,0463</i>	-	-
hsa-miR-451a	0,0496	0,05	0,4075
<b>hsa-miR-92a-3p</b>	<b>0,0842</b>	<b>0,04</b>	<b>0,005</b>
<i>hsa-miR-146a-5p</i>	<i>0,0997</i>	<i>0,16</i>	<i>0,6801</i>

Hsa, homosapiens; adj, adjusted; minus sign (-) analysis was not performed. The miRNAs in bold are significantly differently expressed in both the screening cohort and the validation cohort. The miRNAs in *italics* are down-regulated in patients who die from MCL, miRNAs in normal typeface are up-regulated in patients who die from MCL

## **Legends to Figures**

### **Figure 1**

#### **Flowchart of study**

A dual-method approach consisting of an initial screening by genome-wide miRNA-microarray of MCL samples from the MCL2 cohort, with subsequent confirmation of differentially expressed miRNAs by quantitative real time PCR (qRT-PCR) in the same patients. Confirmed differentially expressed miRNAs identified in the MCL2 cohort were validated by qRT-PCR in samples from the MCL3 cohort.

### **Figure 2**

#### **Heatmap of miRNAs differentially expressed in patients who die from MCL**

Hierarchical clustering of MCL samples from patients of the MCL2 cohort. The microarray generated heatmap demonstrates differentially expressed miRNAs (adjusted p-value <0.05) in patients who died from MCL (cause-specific death) compared to patients who did not die from MCL. 17 miRNAs were found to be differentially expressed; Of these, the 14 miRNAs shown at the top part of the figure had higher expression in the patients who died from MCL, while the three miRNAs shown in the bottom part had lower expression in the patients who died from MCL.

### **Figure 3**

#### **Outcome of MCL patients in the screening cohort (MCL2) according to MIPI-B and MIPI-B-miRNA prognostic models**

The MIPI-B prognosticator (red) divides the cohort into three risk-groups according to established cut-off values. The MIPI-B-miR prognosticator (black), which also includes the qRT-PCR expression level of miR-18b, also divides the cohort into three risk-groups. Kaplan-Meier estimates of (A) cause-specific survival, (B) overall survival and (C) progression-free survival.

#### **Figure 4**

##### **Outcome of MCL patients in the validation cohort (MCL3) according to MIPI-B and MIPI-B-miRNA prognostic models**

Kaplan-Meier estimates of (A) cause-specific survival, (B) overall survival and (C) progression-free survival.

#### **Figure 5**

##### **Outcome of MCL patients in a combined, similarly treated cohort (MCL2 and MCL2) according to MIPI-B and MIPI-B-miR prognostic models**

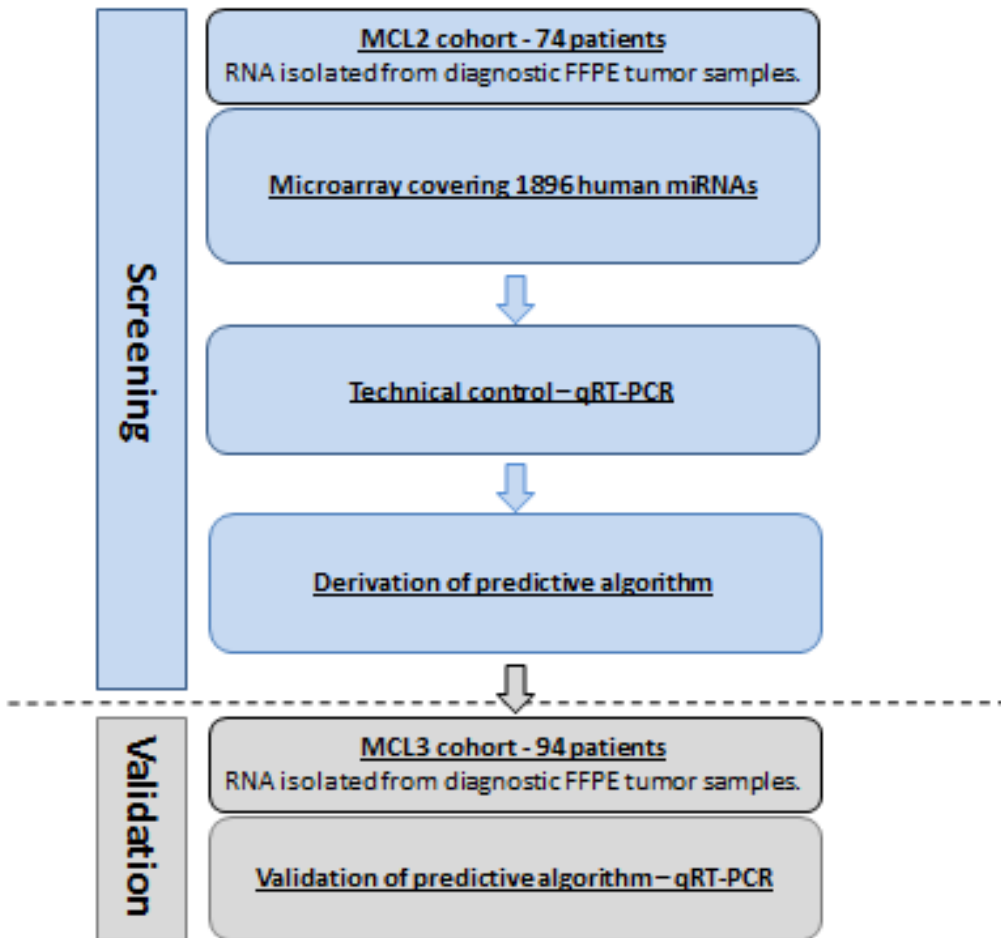
Kaplan-Meier estimates of (A) cause-specific survival, (B) overall survival and (C) progression-free survival.

#### **Figure 6**

MiR-18b inhibits cell proliferation in Jeko-1 and Z-138 cells lines. (A) Relative expression levels of miR-18b in Z-138, Jeko-1 compared to patient MCL samples (PTS) expressing high (PTS High) or low (PTS Low) miR-18b. Results are expressed as means  $\pm$  SD. (B) Cells were transfected with 1  $\mu$ M scrambled (Scr.) miRNA control or miR-18b mimic for 6 days after which cell numbers were determined by XTT assay. Results are expressed as means  $\pm$  SD. Statistical analyzes were performed using Student's t-test. The significance level was set at 0.05. (C) Apoptosis was studied by flow cytometry in Annexin V-PE/7 AAD stained cells 24h after transfection with Scr. miRNA control or miR-18b mimic. (D) Growth curves for Z-138 and Jeko-1 transfected cells. Cell number was determined 0-8 days after transfection using XTT assay. Results are expressed as means  $\pm$  SD.

**Figures**

**Figure 1**



**Figure 2**

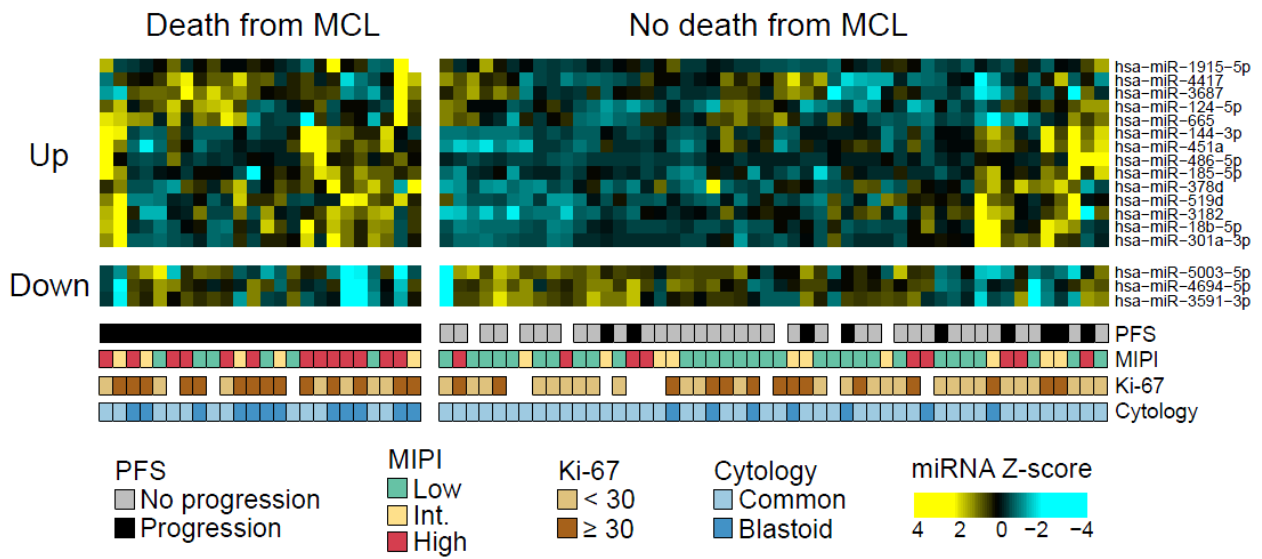
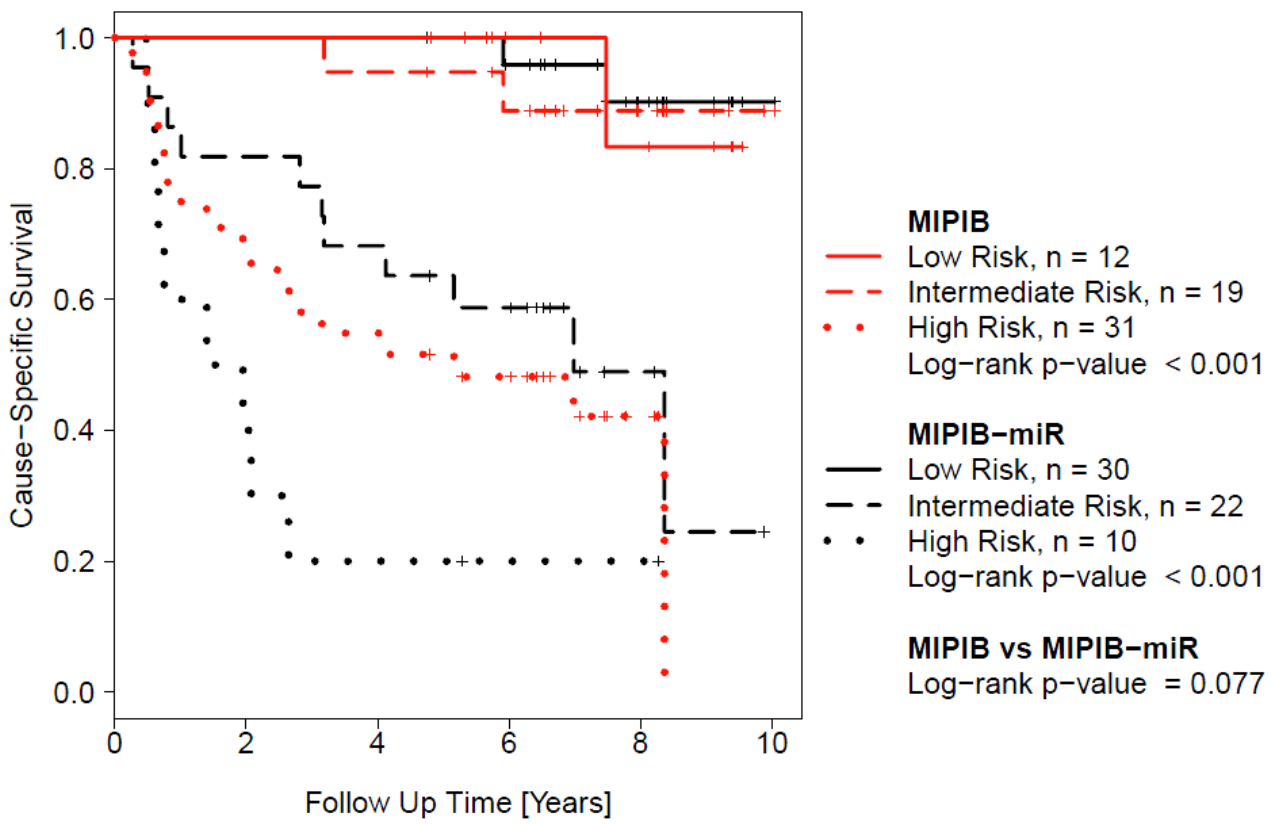
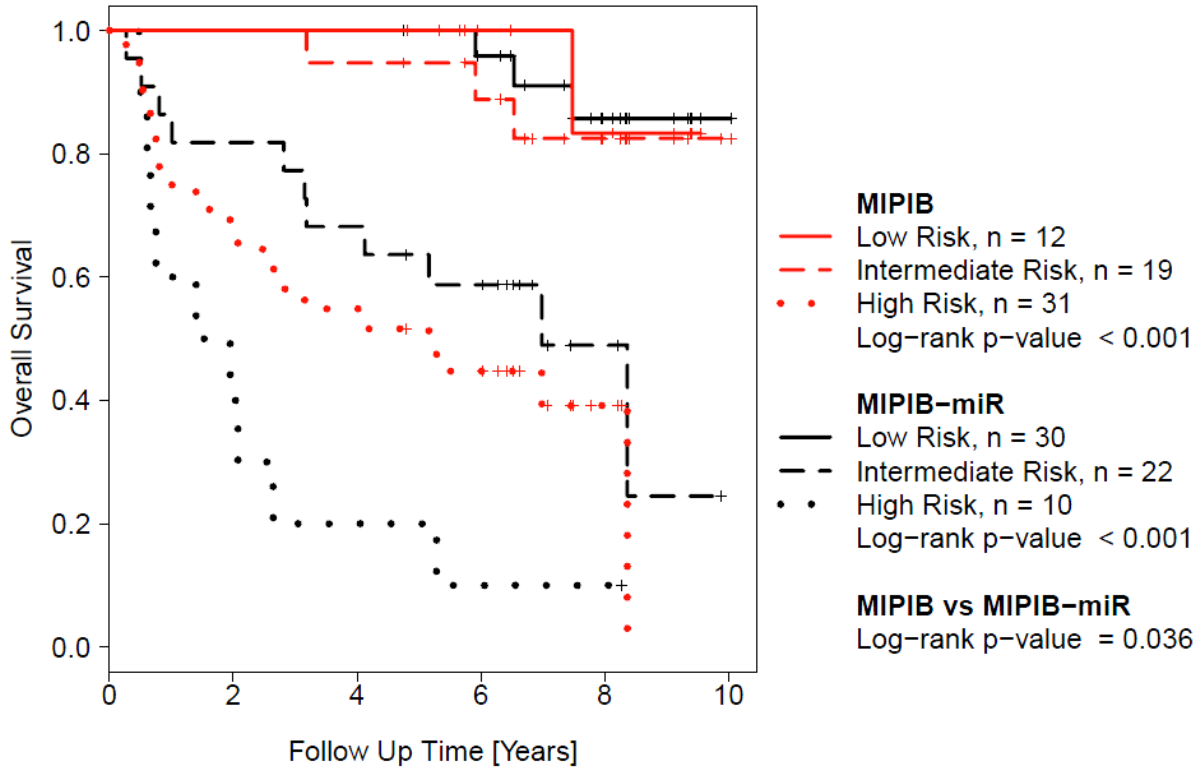


Figure 3

A)



B)



C)

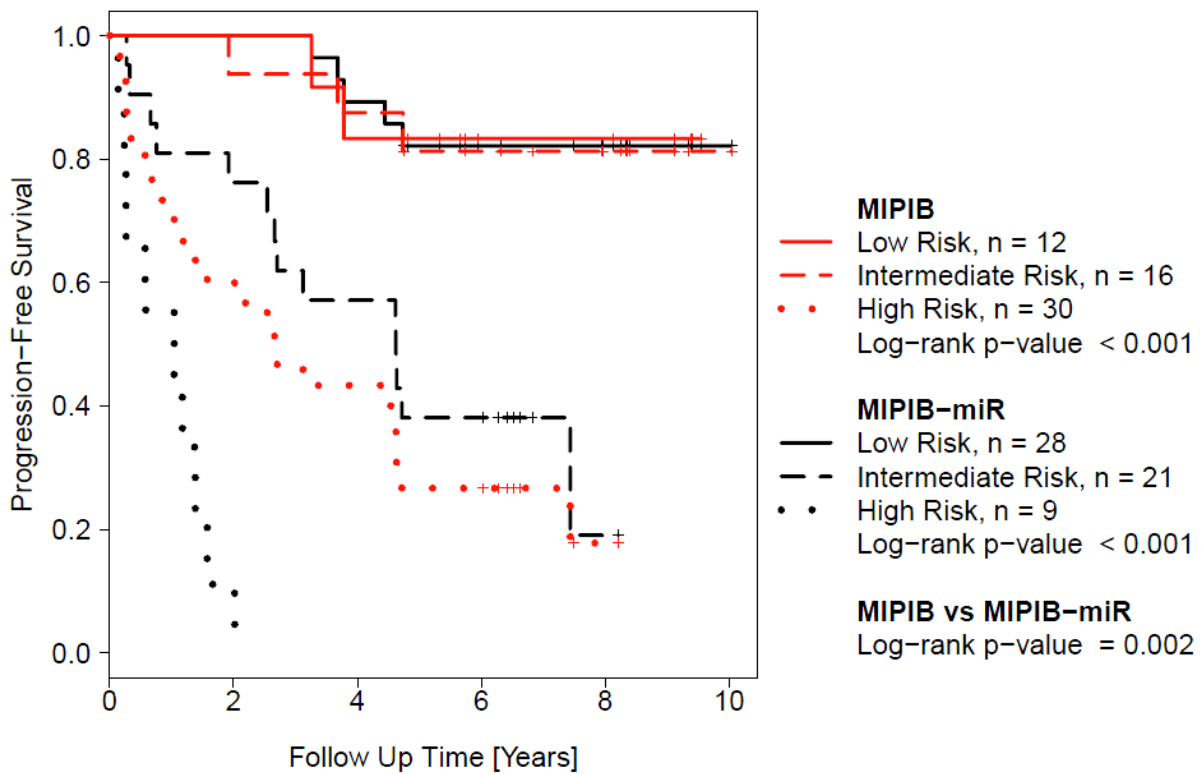
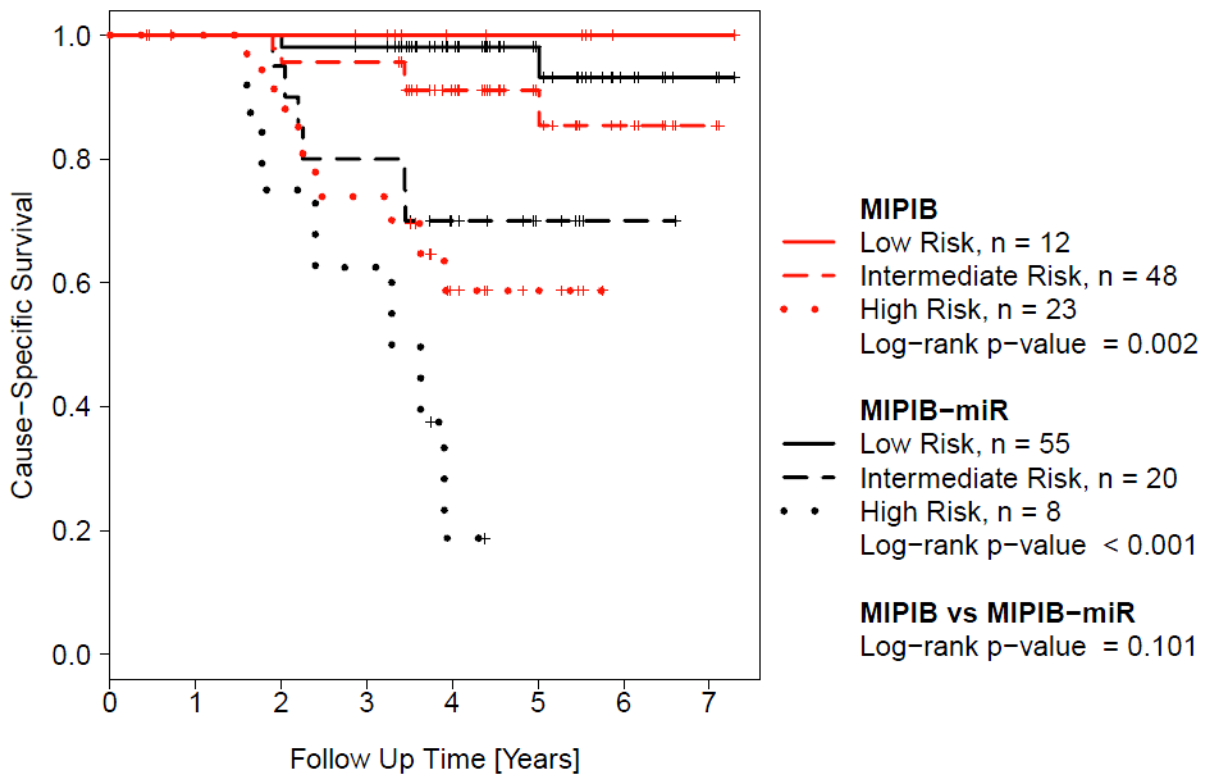


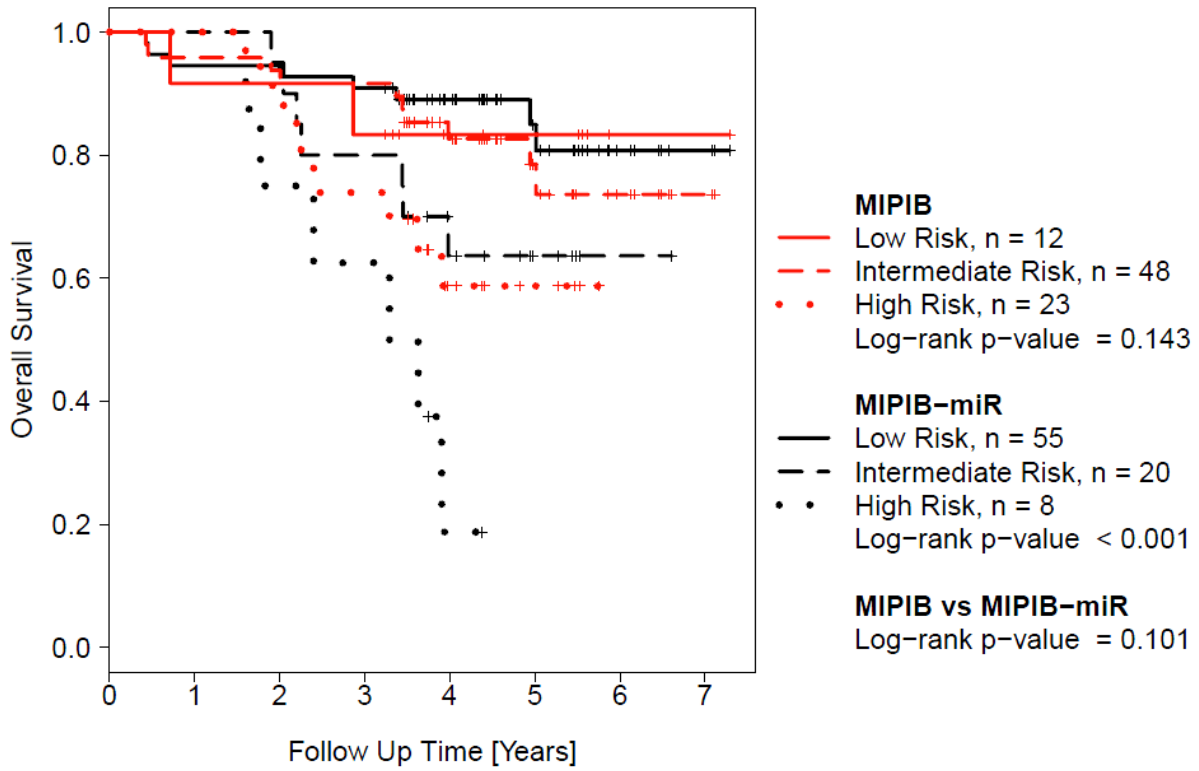


Figure 4

A)



B)



C)

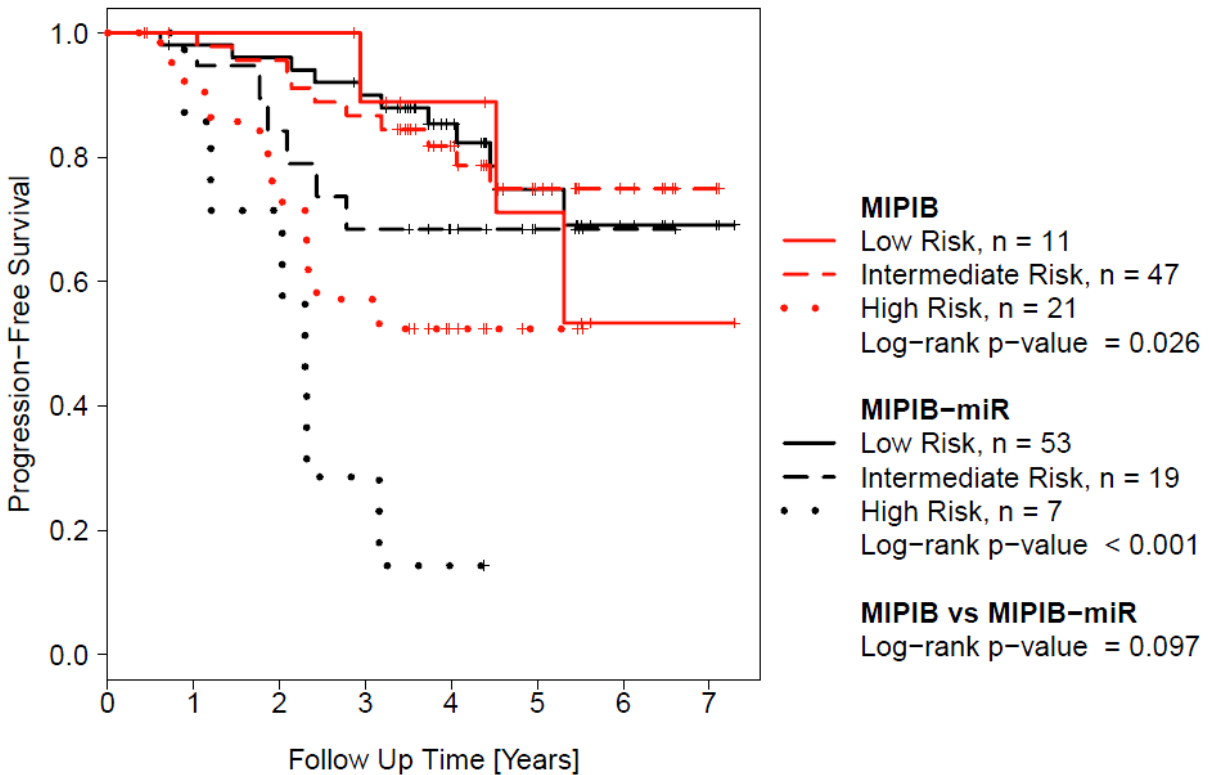
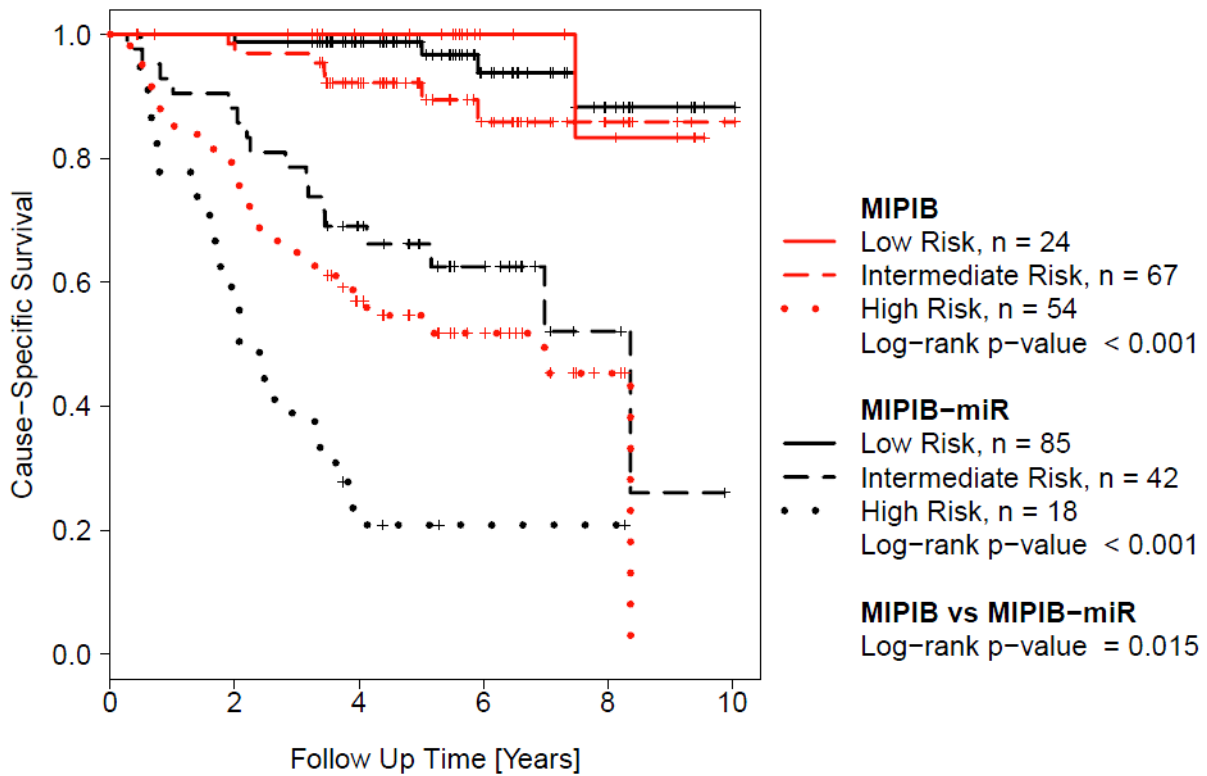
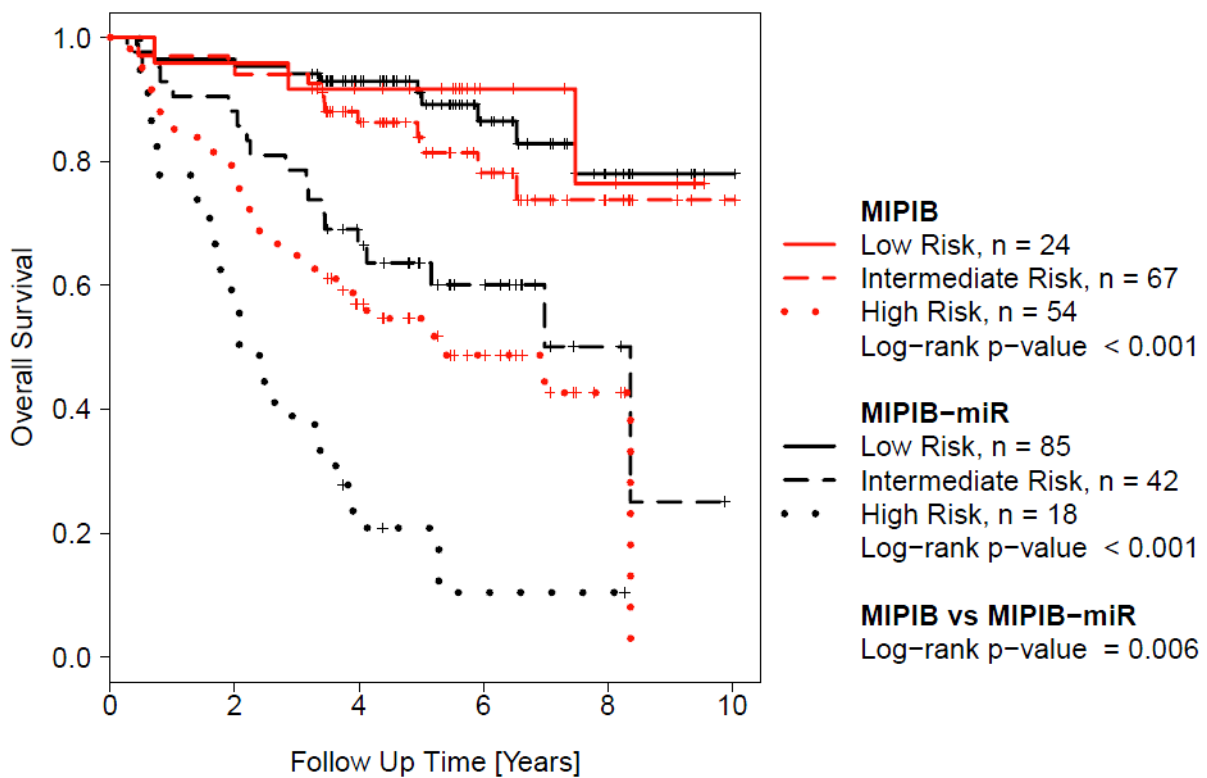


Figure 5

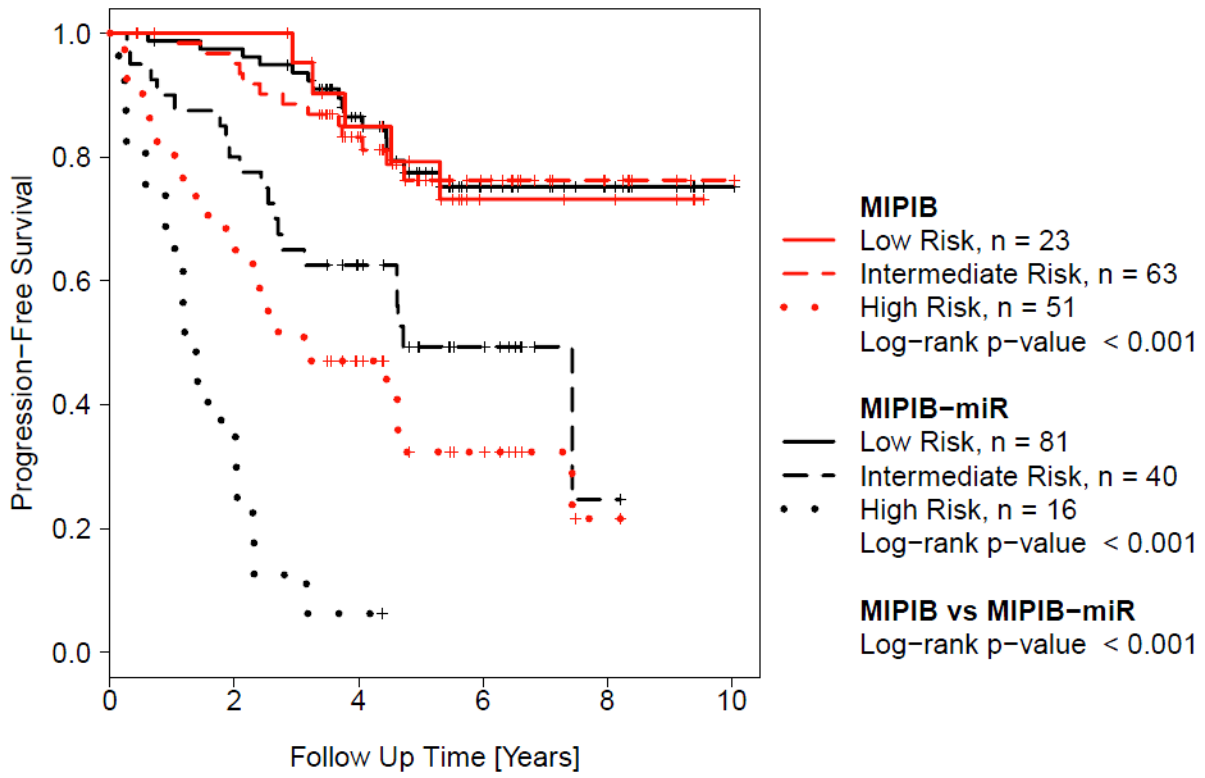
A)



B)



C)



**Figure 6**

