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CHARACTERIZATION OF NON-DRIVER MUTATIONS AND IDENTIFICATION OF

DIFFERENT OUTCOMES AND TREATMENT STRATEGIES BASED ON NGS RESULTS

IN MYELOFIBROSIS PATIENTS IN DIFFERENT CLINICAL STAGES

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Acronyms

Allogeneic HSCT Allogeneic Haematopoietic Stem Cell Transplantation

AP Accelerated Phase

BM Bone Marrow

BP blast phase

CNV Copy-number Variations

CV Confirmed Variants

DDM Data Driven Medicine

DIPSS Dynamic International Prognostic Scoring System

ET Essential thrombocythemia

EFS Event-free Survival

EV Extra Variants

FN False Negative

FP False Positive

Hb Hemoglobin

HMR High molecular risk

IQR Interquartile range

IWG-MRT International Working Group for MPN Research and Treatment

IPSS International Prognostic Scoring System

IQR interquartile range

IWG International Working Group

MF Myelofibrosis

MIPSS70 Mutation-enhanced international prognostic score system

MYSEC-PM Myelofibrosis Secondary to PV and ET-Prognostic Model

MPN Myeloproliferative neoplasm

MPN-SAF TSS Myeloproliferative Neoplasm Symptom Assessment Form total symptom score

MYS SOPHIA Myeloid Solution

"NGS" indicates the 4-tier genomic classification ideated by *Luque Paz et al.* [33]

NGS Next Generation Sequencing

OS Overall Survival

PB Peripheral Blood

Ph neg Philadelphia negative

PMF Primary Myelofibrosis

PV Polycythemia vera

SMF Secondary Myelofibrosis

TN triple negative

TP True Positive

TrN True negative

TTF Time to treatment failure

VAF Variant Allele Frequency

WBC White Blood Cell Count

WHO World Health Organization

Abstract

Myelofibrosis (MF), a chronic Ph-negative myeloproliferative neoplasm, is a clinically and genetically heterogeneous disease. Beside driver mutations that represent the hallmark of pathogenesis, the rapid advancements in gene sequencing technology, like Next Generation Sequencing (NGS), have led to discover additional mutations revealing biological insights in MF and possible novel prognostic markers. However, current clinical prognostic risk-stratification models are the most used in clinical practice. The aim of our project is to use and validate NGS technology in transplant-eligible MF patients, prospectively refining a more reliable prognostic risk assessment and risk-adapted treatment strategy, in real-life setting.

We enrolled 68 MF patients, consecutively diagnosed and followed at Sapienza University. Twenty-two out of 68 (32%) subjects had secondary MF (SMF), diagnosed post essential thrombocythemia and polycythaemia vera. As for driver mutations, 52%, 28% and 3% of patients, carried $JAK2^{V617F}$, $CALR$ and MPL mutation, respectively. One patient had double mutation ($JAK2^{V617F}/MPL$); 10 (15%) patients were identified as triple negative. We found 72 non-driver mutations; 13 out of 68 (19%) patients had a high molecular risk (HMR) profile. The most frequently mutated genes were $TET2$ (n=14, 20%), $DNMT3A$ (n=7, 10%) and $ASXL1$ (n=11, 16%). $ASXL1$ mutated patients carried distinct high-risk clinical features, including higher value of LDH ($p<0.001$), monocytes ($p<0.001$), spleen diameter ($p=0.035$) and symptoms ($p=0.042$). Focusing on mutational profile, no significant differences were detected comparing PMF and SMF. According to the IPSS survival risk distribution at diagnosis in PMF, 32 patients were classified as low risk (70%), 9 as intermediate-1 (20%), 3 as intermediate-2 (6%) and 2 as high (4%). In SMF, the MYSEC-PM risk distribution identified 7 patients as low risk (32%), 13 (59%) as intermediate-1 and 2 as intermediate-2 (9%). The real-life application of MIPSS70 model identified 22 patients, who were previously categorized as low risk according to IPSS/MYSEC-PM, in intermediate risk and allocated 3 patients, previously considered as intermediate risk, in high-risk category. Category shift was due to HMR profile in 7 (10%) patients. Allotransplant was recommended in 5 high-risk patients immediately after NGS results. HMR profile was determinant in proposing transplant choice in 3/9 (33%) intermediate MIPSS70 risk patients. High-risk MIPSS70 category showed inferior OS ($p=0.017$) and EFS ($p=0.005$) than low/intermediate risks. HMR profile negatively influenced overall outcome, both in terms of OS and EFS ($p<0.05$). $ASXL1$ mutated patients had inferior EFS ($p=0.012$) compared to $ASXL1$ wt. These findings were confirmed only in PMF. Moreover, $RUNX1$ mutated patients had significantly shorter OS than $RUNX1$ wt ($p=0.002$). We analysed 21 patients who received ruxolitinib confirming its clinical benefit irrespective of biological findings. Overall, 28 (41%) patients were on clinical treatment-free follow-up. Thirty-nine (57%) patients needed a treatment: 21 (54%) ruxolitinib, 3 (8%) interferon, 14 (36%) hydroxyurea, 1 (2%) allotransplant without a bridge therapy. Globally, 7 patients were allografted. Overall, 4 out of 68 (6%) patients died: 2 due to blast crisis, 1 for transplant complication and 1 for SARS-CoV-2 infection in MF progression.

In our monocentric prospective real-life study, NGS analysis allows a better risk stratification and a more accurate risk-adapted therapy of MF patients, contributing to characterize mutational landscape of the disease.

1. Introduction

1.1 Definition and epidemiology

Myelofibrosis (MF) is a Philadelphia negative (Ph neg) chronic myeloproliferative neoplasm (MPN), an acquired clonal hematopoietic stem cell disorder characterized by the abnormal proliferation and accumulation of mature blood cells, bone marrow dysregulation stroma and development of reticulin and/or collagen marrow fibrosis, osteosclerosis, and extramedullary haematopoiesis. MF can present de novo as primary myelofibrosis (PMF) or it can arise as a secondary process from antecedent disease, like essential thrombocythemia (post-ET MF) or polycythemia vera (post-PV MF) [1]. Current diagnosis of PMF is based on the 2016 World Health Organization (WHO)-criteria (Table 1)[1,2], largely unchanged in the last revision [3], which underlined the importance of distinguishing prefibrotic/early (pre-PMF) from ET as well as from fibrotic PMF (overt PMF) [3]. Indeed, overt-PMF differs not only for morphological features but also for pronounced disease manifestations, worse outcome and major risk of leukemic transformation than pre-PMF [3,4].

Diagnostic criteria of secondary myelofibrosis (SMF) have been established by the International Working Group for MPN Research and Treatment (IWG-MRT) [1,5] (Table 2).

MF is a rare disease; the incidence of PMF in Europe varies from 0.1 to 1/100.000 [5]; the 15-year cumulative incidence of MF evolution rates varying between 5% and 19% for PV and between 4% and 11% for ET [7]. Median age at diagnosis is 68 years [8, 9]. Male predominance was reported for both PMF and post-PV MF, but not for post-ET PMF [10].

MF is clinically and genetically heterogeneous disorder with a highly variable survival, ranging from 2 to 11 years [11]. Leukemic progression occurs in about 20% of patients, representing one of the most frequent causes of death [1,11]. Mortality was also determined by comorbid conditions like cardiovascular events, infections, or bleeding as consequence of cytopenia. Allogeneic

hematopoietic stem cell transplantation (allogeneic HSCT) remains the only curative treatment, but it is not feasible in all patients due to the high risk of complication and mortality [1].

1.2 Pathogenesis

The exact pathogenesis of MF and in general of MPN is not fully understood [12]. An undoubted hallmark is the constitutional activation of JAK-STAT pathway driven by acquired somatic mutations in myeloid progenitor cells. Phenotypic driver mutations have been identified in *JAK2*, *CALR* and *MPL* genes and occur in a mutually exclusive manner, with only infrequent co-occurrence (1–2% of cases) [8]. *JAK2* V617F is the most frequent, being found in approximately 55-65% of PMF, followed by *CALR* in 20-30% and *MPL* 5-10%. Less than 10% of patients are defined "triple negative" (TN), because do not express any one of the three driver mutations [12,13].

JAK-STAT pathway is involved in several cellular processes, orchestrating cell proliferation, stem cell maintenance, differentiation and regulation of the immune system. Normally, the bind of ligand to type I cytokine receptors including thrombopoietin (TPO) receptor MPL, colony-stimulating factor (G-CSF) receptor, and erythropoietin (EPO) receptors leads to activation of JAK and phosphorylation of STATs. The dimerization and translocation of STATs to the nucleus determine regulation of gene transcription of the myeloid lineage cells [12,13]. The *JAK2* V617F is a point mutation in exon 14 of the *JAK2* gene that causes a single amino acid (valine to phenylalanine) substitution and conformational change in the JH2 pseudo-kinase domain of JAK2. The loss of normal inhibitory function of the JH2 domain leads to constitutive activation of the JAK-STAT pathway irrespective of EPOR, MPL or G-CSFR ligand binding [12,13]. The majority of *JAK2* V617F negative MF patients have detectable mutations in *MPL* or *CALR.* TPO and its receptor, MPL, plays a crucial role in megakaryocyte differentiation and maintenance of hematopoietic stem cells. In *MPL* mutated cases, the absence of its regulation function on TPO, causes elevated plasma TPO levels and thus, an

uncontrolled megakaryocytopoiesis. The most frequent *MPL* mutations are W515L and W515K types, in the exon 10 [12-14].

CALR mutations were reported for the first time in 2013 by two different research groups [16,17]. The wild type *CALR* is an endoplasmic reticulum (ER) chaperone protein, involved in calcium homeostasis, regulation of protein quality control and other processes (immune response, transcription, cell adhesion/migration, cell proliferation and immunogenic cell death). It is structurally constituted of 3 domains: N-domain responsible for chaperone-like function, an arm-like structure P-domain and a C-terminal acidic domain, which contains a KDEL motif responsible for the ER retention signal. The mechanism by which CALR is implicated in the pathogenesis of MPN is more elusive than the other driver mutations, *JAK2* V617F and *MPL* [12]. However, several studies demonstrated that *CALR* mutated and MPL interaction is based on positive charge on the C-terminal domain of CALR mutant, possible responsible of the inactivation of P-domain and the binding of N-domain to MPL. This interaction probably alters MPL structure in a fashion similar to that induced by its engagement by TPO, inducing cytokine-independent growth [17]. To date, more than 50 different *CALR* mutations have been identified in exon 9 and are classified in type 1 (c.1092_1143del, L367fs*46, 52-bp deletion) or type 2 (c.1154_1155insTTGTC, K385fs*47, 5-bp insertion). In a minority of patients type1-like or type2-like *CALR* mutations were found [13]. Even the driver mutations are crucial in MPN biology, they also have a prognostic role. In fact, MF patients with *CALR* type1/type1-like mutations have better survival probability than patients with *JAK2* V617F or *CALR* type 2/type2-like or *MPL* mutations whereas TN cases have the worst outcome [18,19].

Other mechanisms seem to be a key features in the MPN pathogenesis, affecting phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways that could be collaterally activated by JAK2 mutant, independently of STATs [12]. Furthermore, inflammatory states of MF is also currently under investigation. The increases in circulating levels of cytokines,

chemokines and reactive oxygen species contribute to genetic instability and, thus, could be determinant in clinical manifestation and pathogenesis of MF [20]. Hyperactivation of TGF-β, PDGF and FGF promote fibrosis, neo-angiogenesis and osteosclerosis [21]. Further investigation on the role of microenvironment, hematopoietic niche and inflammation state of MF are needed not only for understanding pathogenesis but also for the implementation of novel therapeutic target.

1.3 Next-generation sequencing and non-driver mutations

Next generation sequencing (NGS) enables a massive sequencing of DNA allowing the simultaneous evaluation of multiple genes. It consists of four steps: library preparation, cluster generation, sequencing and data analysis. The undoubted advantage of NGS compared to different sequencing (es. Sanger Sequencing) methods is its high-throughput property to discover concomitant mutations in different samples in the same run, starting from a relatively low input of DNA/RNA. In addition, NGS can simultaneously screen several genomic aberrations, such as single/multiple nucleotide variants (SNVs), small and large insertions and deletions (INDEL) and copy-number variations (CNV), with high sensitivity and accuracy [22]. Since NGS technology developed, other somatic mutations have been increasingly identified in other than *JAK2/CALR/MPL* genes, mainly involving in signal transduction (*CBL, KRAS, NRAS, PTPN11* and *LNK/SH2B3*), transcription activity (*RUNX1, NFE2*), chromatin modification (*TET2, EZH2, IDH1/2, ASXL1* and *DNM3TA*), RNA splicing (*SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*) and tumor suppressor function (*TP53*) [23,24]. The use of NGS assay in clinical practice is increasing and aims to elucidate the clonal nature of the disease (especially in TN cases) and better refine prognosis. Additional mutations can occur before, after or concomitant the acquisition of MPN driver mutation, influencing clinical phenotype and clinical course.

The most frequent mutated genes in PMF include *ASXL1* (13-40%), *TET2* (10-20%), *SRSF2* (9-19%), *DNMT3A* (7%) and *EZH2* (5%), while mutations in *IDH1/IDH2*, *TP53* and *LNK* seem to be less frequent

in chronic phase than in blast phase [8,25]. Similarly, *RUNX1* and *RAS* mutations are rarely present at the time of diagnosis but preferentially appear at the time of leukemic transformation [25]. *ASXL1*, *SRSF2*, *EZH2*, *IDH1-2* mutations have been correlated with worse prognosis and have been defined as high molecular risk (HMR) mutations in PMF patients [26]. Furthermore, carrying at least 2 mutations in *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2* is associated with a significantly shorter survival (median 2.6 years) compared to one mutation (median 7.0 years) or no mutations (median 12.3 years), irrespective of clinical adverse features [27]. Mutations in *EZH2*, *ASXL1* [28] and *IDH1-2* seem to negatively impact leukemia-free survival [29].

Recently, *U2AF1* mutations have been associated with cytopenic phenotype and worse survival [30]. Aberrations affecting other genes as *CBL/NRAS/KRAS* seem to be instead implicated in treatment resistance and associated with poor prognostic features [31]. Overall, the role of non-driver mutations in patients with SMF is less clear than PMF. Although HMR mutations seem to occur with roughly similar frequencies in SMF, they have shown less impact on survival in SMF than PMF patients [8,32]. In addition, the prognostic role of *ASXL1* is still on debate, especially in SMF setting [32]. Luque Paz et al. [33] argued for a revision of the prognostic classification of somatic mutations in MF, suggesting the inclusion of *TP53*, *U2AF1*, *CBL*, *NRAS*, and *KRAS* genes to the already-recognized HMR mutations and the exclusion of *ASXL1* gene as mutations in this latter seem not to have prognostic impact alone, but only when associated with high-risk mutations. The authors proposed a novel model, named "NGS", including 4 genetic categories: *TP53* mutated, high risk (at least 1 mutation in *EZH2*, *CBL*, *U2AF1*, *SRSF2*, *IDH1*, or *IDH2*), *ASXL1* mutated only, and others [33].

1.4 Clinical manifestations and risk assessment

Approximately 30% of patients are asymptomatic at diagnosis. Main clinical manifestations of MF include anemia, marked hepato-splenomegaly with abdominal discomfort, constitutional symptoms

(eg, fatigue, night sweats, fever), cachexia, bone pain, pruritus, thrombosis, and bleedings. Other possible complications are related to symptomatic portal hypertension, that can lead to variceal bleeding or ascites, and extramedullary haematopoiesis as cord compression, ascites, pleural effusion, pulmonary hypertension, or extremity pain [1,11,25]. Symptoms may impair quality of life, functional status and activities of daily living. The MPN-SAF Total Symptom Score (MPN-SAF TSS) is a concise and objective tool useful to assess MF-associated symptoms, exploring the most relevant symptoms in patients with MPN (fatigue, concentration, early satiety, inactivity, night sweats, itching, bone pain, abdominal discomfort, weight loss, and fever) [34,35].

The natural history of patients with MF is variable. In the last decade, several prognostic scores have been developed to predict survival probability (Table 3). The International Prognostic Scoring System (IPSS) [36] was the first model developed for PMF and the most widely used in real-life practice [37]. It includes five independent predictors of inferior survival (age >65 years, hemoglobin (Hb) <10 g/dL, leukocyte count >25×10^9/L, circulating blasts ≥1% and presence of constitutional symptoms) and categorizes patients into 4 prognostic groups: low risk, intermediate-1, intermediate-2, and high risk; the corresponding median survivals were reported at 11.3, 7.9, 4, and 2.3 years, respectively [1,36].

The IWG-MRT subsequently developed a dynamic prognostic model (DIPSS) [38] that can be applied at any time during the disease course. DIPSS takes into account the same prognostic variables used in IPSS but assigned two, instead of one, adverse points for Hb value <10g/dL, identifying low (0 points), intermediate-1 (1-2 points), intermediate-2 (3 or 4 points) and high (5-6 points) risk categories. Furthermore, the addition of other 3 factors (platelet count <100×10^9/L, red cell transfusion needs and unfavourable karyotype) led to the development of DIPSS-plus prognostic score [39]. Unfavourable karyotype is defined as follows: complex karyotype or one or two

abnormalities that include trisomy 8, del 7/7q, i(17q), del5/5q, del12p, inv(3), or 11q23 rearrangement [39].

Recently, the Mutation-enhanced international prognostic score system (MIPSS70) [40] was developed in PMF patients aged 70 years or younger and integrates biological insights and clinical features, so incorporating three genetic [absence of *CALR* type-1/like mutation; presence of high molecular risk mutations (*ASXL1*, *SRSF2*, *EZH2*, *IDH1-2*) and presence of ≥2 high molecular risk mutations] and six clinical risk factors (hemoglobin <10 g/dL; leucocytes >25 × 10^9/L; platelets <100 × 10^9/L; circulating blast ≥2%; bone marrow fibrosis grade ≥ 2, and constitutional symptoms). It stratifies patients into three risk categories (low risk, intermediate risk and high risk), with corresponding median survival ranges of 27.7 years -'not reached', 6.3-7.1 years and 2.3-3.1 years, and was validated in 2 independent cohort [40].

MIPSS70+ version 2.0 includes also the three tiered cytogenetic risk levels, *U2AF1* Q157 as HMR mutation and sex and severity-adjusted Hb thresholds [41], individualizing five risk categories: very high risk, high risk, intermediate risk, low risk and very low risk, with median survivals of 1.8 years, 4.1 years, 7.7 years, 16.4 years and "not reached", respectively. GIPSS [42] is instead a prognostic model that is exclusively dependent on mutations and karyotype.

Although the prognostic scoring systems described above are clinically used in SMF, the Myelofibrosis Secondary to PV and ET–Prognostic Model (MYSEC-PM) [43] represents the most specific tool for disease prognostication in SMF. It includes 6 independent predictors of inferior OS: 2 points for a Hb level less than 11 g/dL, a circulating blast percentage of at least 3%, and an unmutated *CALR* genotype; 1 point for a platelet count less than 150 × 10^9/L and constitutional symptoms; and 0.15 point for any year of age.

1.5 Treatment

1.5.1 Allogeneic stem cell transplantation

To date, as aforementioned, allogeneic HSCT is the only potentially curative treatment option in MF patients [1].

Reduced-intensity conditioning (RIC), better HLA donor selection, graft versus host-disease (GVHD) and anti-infective treatments, the use of JAK-inhibitors to reduce splenomegaly led to extending the feasibility and safety of allogeneic HSCT. However, transplant is still associated with high rate of morbidity and mortality [1]. Thus, the assessment of risk in order to individualize patients who likely benefit from allogeneic HSCT is crucial, balancing the risk of the procedure against expected survival without allogeneic HSCT. Bridging therapy can be used to decrease symptoms burden and splenomegaly before transplant, as spleen size could impair engraftment and survival probability [44].

In 2015, the European LeukemiaNET/EBMT expert consensus suggested that patients with intermediate-2 or high-risk disease according to the IPSS, DIPSS or DIPSS-plus and age <70 years or patients <65 years with intermediate-1 risk and adverse features (transfusion-dependent anemia, >2% of peripheral blasts, adverse cytogenetics) should be considered potential candidates for allogeneic HSCT [45].

Subsequently, some authors suggested that triple negative disease or the presence of HMR mutations could represent a trigger for an early transplantation even if patients belong to lower risk classes [44]. Indeed, allogeneic HSCT seem to overcome the adverse prognostic significance of additional mutations [46].

However, in real-life setting, most patients with MF are not eligible for transplantation and their treatment is only focused on symptomatic control and prevention of disease complications.

On the other hand, patients at low risk or intermediate-1 risk without disease-related manifestations (symptoms, anemia, splenomegaly, leucocytosis, marked thrombocytosis) do not benefit of any treatment and so observation alone is a reasonable approach [1].

1.5.2 Management of MF-Associated Anemia

Erythropoiesis stimulating agents aims to improve anemia in case with inadequate serum erythropoietin levels [8]. Rate of responses is around 40% and the median response duration is approximately around 12 months [8,47]. Negative predictors of response are elevated serum ferritin levels at baseline and red blood cell (RBC) transfusion dependence [48,49]. Other options for treating anemia are danazol and immunomodulatory agents (lenalidomide, thalidomide, and pomalidomide) [11]. As anemia could be related to increased TGF-β, luspatercept is currently under exploration in phase III trial for patients with RBC transfusion-dependent MF on JAK2 inhibitor therapy (NCT04717414)[50,51].

1.5.3 Management of hyperproliferative manifestations of MF

Hydroxyurea

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, is an effective treatment option for the hyperproliferative manifestations of MF (thrombocytosis or leukocytosis) and it is especially used in lower risk patients resulting in clinical improvement in 40% of patients [52]. The major toxicities manifestation are anemia, pancytopenia and ulcer formation [11].

Interferon

Historically, the use of interferon-α (IFN-α) has been limited due to its toxicity. Pegylated formulations are more tolerable and manageable. However, interferons are generally not

recommended for higher-risk disease. In a French cohort of 62 MF patients [53], pegylated IFN decreased the *JAK2* V617F allele burden by >50% in a consistent proportion of patients and the long-term updates results showed that the presence of HMR abnormalities reduced treatment responses [54]. As its possible role in reducing fibrosis and allele burden, the combination of IFN-α and jak-inhibitors, which are not disease *modifying* agents, is investigated. Preliminary results from the phase I/II RUXOPEG trial revealed that the combination is effective in reducing spleen size and *JAK2* V617F allele burden in MF patients [55].

1.5.4 JAK inhibitors

Ruxolitinib

Ruxolitinib is the first-in-class JAK1/JAK2 inhibitor approved for MF patients, based on the results of phase III studies (COMFORT-I and COMFORT-II) [56,57]. It induced spleen size reduction and MF-related symptom improvements compared with placebo (COMFORT-I) [56] or best available treatment (COMFORT-II) [57]. In the COMFORT-I, the rate of patients reaching the primary endpoint of spleen reduction (≥35% reduction in spleen volume as assessed by MRI at 24 weeks) was significantly higher in ruxolitinib group compared to placebo group (42% vs 0.7%, respectively). In addition, an improvement of at least 50% in the MF-SAF at 24 weeks was seen in 46% of patients treated with ruxolitinib (vs 5% of placebo group). [56]. In the COMFORT-II, the primary endpoint (≥35% reduction in spleen volume as assessed by MRI or CT scan at 48 week) was reached in 28% of patients in ruxolitinib group as compared with 0% in the BAT group. The clinical benefit of ruxolitinib was observed irrespective of patients subgroups (PMF, SMF, IPSS risk groups, JAK mutation status, intermediate-2 and high risk) [57].

The 5-year follow-up analysis of COMFORT-I and COMFORT-II confirmed the efficacy of ruxolitinib [58,59]. Furthermore, the exploratory analysis of 5-year data pooled from these trials demonstrated

that ruxolitinib prolonged survival than control arm in intermediate-2 and high-risk MF, even if the trials were not powered to show impact on overall survival (OS) [60]. A propensity score matching analysis of the ERNEST study, based on prospectively collected real-world data, demonstrated a survival advantage for patients treated with ruxolitinib in first or second-line than HU treatment (median OS 7.7 vs 3.5 years, respectively) [61]. However, some authors retain whether ruxolitinib improves survival in myelofibrosis remains an unmet urgent clinical need as randomized controlled trials specifically addressed to this issue and to the direct comparison between HU and ruxolitinib are lacking [62]. Open questions also remain the definition of resistance criteria, suboptimal response, timing of transplant and the management of patients after ruxolitinib interruption [37]. Anemia and thrombocytopenia are the most common hematologic ruxolitinib-related toxicities, particularly occurring during the first 8 to 12 weeks of treatment [58,59,63]. Ruxolitinib is also associated with a potentially increased risk of opportunistic infections, viral reactivation and non-melanoma skin cancer. In COMFORT-I and COMFORT-II studies [58,59], the rate of treatment discontinuation was approximately 50% at 3 years and 75% at 5 years; 35% of patients, including intermediate-1 risk category, discontinued ruxolitinib after 3 years in the JUMP study [63]. Real-life experiences confirmed these data, highlighting a rate of discontinuation due to failure or intolerance of approximately 50% at 3 years, with subsequent dismal prognosis, especially for patients who lacked or lost a spleen response [37,64]. Higher DIPSS risk category, low platelet count, unfavourable karyotype and RBC transfusion dependency at ruxolitinib start seem to increase the probability of drug discontinuation [64]. Recently, the RR6 model [65] was proposed and validated in real-life MF cohort [66] for the early identification of RUX-treated MF patients with impaired survival for whom a prompt treatment shift could be reasonable. The model included only clinical factors, like a reduced ruxolitinib dose at baseline, 3 and 5 months, RBC transfusion need at months 3 and/or 6 and at all time points [65]. Regarding the possible impact of mutational signature on ruxolitinib

efficacy, a subanalysis of COMFORT-II study showed that the likelihood of obtaining spleen size reduction and symptoms improvement was not affected by the presence of HMR; ruxolitinib seems to mitigate the negative prognostic impact of mutations [67]. Conversely, some studies reported that the presence of one or more HMR mutations at baseline or acquired during treatment have been associated with increased risk of ruxolitinib resistance [8]. *RAS/CBL* mutations were associated with a lower probability of obtaining symptoms and spleen responses in 61 jak-inhibitors treated patients [31]. Future investigations are needed to better understand the biological mechanisms of ruxolitinib resistance, including the role of non-driver mutations.

Fedratinib

Fedratinib, a selective JAK2 and FLT3 inhibitor, was recently approved in both JAK inhibitor naïve and ruxolitinib pre-treated patients for the management of symptoms and splenomegaly. In the Phase 3 JAKARTA study, fedratinib was superior to induce spleen and symptoms response than placebo group in JAK inhibitor naïve patients with MF [68]. Efficacy was also assessed in the phase 2 JAKARTA2 trial which included patients with intermediate- or high-risk MF who failed ruxolitinib treatment [69]. Indeed, spleen and symptoms response were reached in 55% and 26% of patients, respectively. The most common drug-related toxicity are anemia, thrombocytopenia and gastrointestinal toxicities (diarrhea, vomiting and nausea). Initially, Drug Administration (FDA) placed fedratinib on clinical hold because of suspected cases of Wernicke encephalopathy, promptly lift after additional safety data, confirming fedratinib does not increase the risk of thiamine depletion. Assessing nutritional status and thiamine levels are recommended in prescribing information [70].

Pacritinib

Pacritinib is a JAK2-selective and IRAK-1 inhibitor, and it is FDA-approved for the treatment of intermediate or high-risk MF with a platelet count less than 50 x 10^9/L. In the phase III PERSIST-1 trial [71], patients without prior JAK inhibitor exposure were randomized 2:1 to pacritinib or best available therapy (BAT) excluding ruxolitinib, irrespective of baseline platelet, whereas in the PERSIST-II trial [72], MF patients with baseline thrombocytopenia irrespective of prior ruxolitinib therapy were randomized 1:1:1 to pacritinib 400 mg daily, pacritinib 200 mg twice daily, or BAT including ruxolitinib. Both trials assessed the efficacy of pacritinib, even in patients with severe baseline thrombocytopenia (platelet count $<50 \times 10^9$/L), thus representing a new therapeutic option for myelodepletive phenotype [71,72].

Momelotinib

Momelotinib is a JAK1/JAK2 inhibitor. It also inhibits ACVR1, causing downregulation of hepcidin transcription and increase iron availability for erythropoiesis [73]. In preliminary data of the pivotal phase 3 MOMENTUM trial [74], momelotinib have been providing significant improvements in symptoms, spleen size and anemia vs danazol in patients with symptomatic and anemic myelofibrosis who were previously treated with JAK inhibitor. Momelotinib will take on a critical role in addressing unmet medical need of MF patients with moderate/severe anemia.

1.5.5 Novel drugs

Several novel agents are under investigation in monotherapy or in association with JAK-inhibitors, attempting to eradicate the disease, ameliorate fibrosis and overcome the limitations of JAK-inhibitors. Examples of promising novel agents are pelabresib (bromodomain and extra-terminal protein inhibitors, bomedemstat (lysine-specific (histone) demethylase-1 inhibitor), navitoclax

(inhibitor of the anti-apoptotic B-cell lymphoma-2/extra-large family of proteins), parsaclisib (selective inhibitor of PI3K delta), imetelstat (inhibitor of telomerase activity) [75].

1.6 Response criteria and follow-up

MF patients need close clinical and laboratory follow-up. The Revised IWG-MRT and European LeukemiaNet (ELN) [76] consensus report defined 6 response categories (Table 4), recommending to monitor anemia response, spleen response and symptom response or every sign of disease progression every 3 to 6 months during the course of treatment. However, results of a recent real-life survey showed that criteria to evaluate spleen response were differently defined among physicians, concerning both the entity of size reduction and the modality of evaluation (clinical or radiological exams). As previously mentioned, also uniform criteria for ruxolitinib treatment failure have to be still clarified [37].

2. Rationale of the study

MF is the most aggressive of the Ph-negative MPN disease and it is characterized by clonal proliferation of myeloid cells, extramedullary haematopoiesis, splenomegaly, progressive bone marrow fibrosis and systemic symptoms, with impaired quality of life and survival. The natural history of MF is also affected by an increased risk of thromboembolic and/or haemorrhagic events and leukemic transformation. Survival is highly variable, ranging from months to many years [1].

Although the availability of JAK1/2 inhibitors has significantly advanced MF treatments, these are not disease-modifying agents, thus allogeneic HSCT still remains the only curative treatment option. Several clinical prognostic risk scores have been developed to predict survival and identify patients who could really benefit from transplant strategy.

Beside driver mutations that represent the hallmark of pathogenesis, the rapid advancements in gene sequencing technology, like NGS, have led to discover additional mutations revealing biological insights in MF and possible novel prognostic markers. New prognostic scores have been assessed by the integration of biological and clinical features, as MIPSS70, MIPSS70 v2 etc. However, current clinical prognostic risk-stratification models are the most used in clinical practice. The aim of our project is to use and validate the NGS technology in transplant-eligible MF patients, prospectively refining a more reliable prognostic risk assessment and risk-adapted treatment strategy, in real-life setting.

Specific aims of our project include:

1. Validation of NGS workflow on the Illumina MiSeq platform

2. Correlation between driver mutations and clinical phenotype

3. Correlation between non-driver mutations and clinical findings (hematological parameters, grade of fibrosis, splenomegaly and symptoms)

4. Differences between PMF and SMF

5. Differences in terms of outcome according to the type and the number of non-driver mutations

6. Correlation between non-driver mutations and response to JAK2 inhibitor

7. Correlation between HMR profile and outcome in terms of overall survival (OS), event-free survival (EFS) and time-to-treatment failure (TTF)

8. Identification of different prognostic subgroup and a subsequent risk-adapted treatment strategy

3. Material and methods

3.1 Validation experiment

We performed two independent NGS runs of 8 samples each, with 3 inter-run and 1 intra-run replicates. Samples with confirmed variants (CVs) with other assays and characterized regions provided by SOPHia GENETICS (SG) were used to assess the analytical performance of our workflow, including sensitivity, specificity, accuracy, precision, repeatability, and reproducibility. True positive (TP), false positive (FP), extra variants (EV), true negative (TrN) and false negative (FN) values are defined as follows:

-TP are CVs detected by SOPHiA Data Driven Medicine (DDM®)

-FN are CVs not detected by SOPHiA DDM

- FP are non-CV detected by SOPHiA DDM within characterized regions

- EV are non-CV detected by SOPHiA DDM in uncharacterized regions

- TrN position in characterized region with no variant detected an no confirmed variant specified.

Sensitivity is calculated as the percentage of CVs detected [TP/(TP + FN)*100]; specificity is determined as the percentage of negative positions that were correctly identified as negative [TrN/(TrN + FP)*100]. Accuracy is defined as percentage of correct calls (positive and negative) [(TP+TrN)/(TP+FN+FP+TrN)*100]; precision is calculated as percentage of correct positive calls from all positive calls [TP/(TP+FP)*100]. Sequencing Repeatability (SR) is determined, for each pair of intra-run replicates A and B, as the percentage of bases that are well-defined between samples (intersection of well-defined bases) among all bases well-defined in either sample (union of well-defined bases). Bases are considered well-defined if they are sufficiently covered and do not contain low confidence variant calls. Variant Repeatability (VR) is the fraction of variants that are identical

in both replicates among all the bases that are well-defined in both replicates. Repeatability is defined as the product of SR and VR. Reproducibility is calculated equivalently to Repeatability for inter-run replicates.

3.2 Study cohort

We evaluated 68 patients who were consecutively diagnosed with MF according to 2016 WHO classification (PMF) [2] and IWG-MRT (SMF) [5] criteria at our Institute of Haematology, Sapienza University of Rome, between 2018 to January 2022. All patients were <65 years and potential candidate for allogeneic HSCT. Baseline and follow-up data were prospectively and anonymously collected in a dedicated database. We used the Myeloproliferative Neoplasm Symptom Assessment Form total symptom score (MPN-SAF TSS) [34] to provide an accurate assessment of symptoms in all patients. Disease-related symptoms were also recorded as categorical variable (presence/absence). Spleen size was evaluated by physical examination and abdomen ultrasound. IPSS and DIPSS [36,38] were calculated for all PMF, MYSEC-PM [43.] risk was assessed for SMF. MIPSS70 risk was assessed for all patients [40] after NGS analysis. Cytogenetic analysis could be feasible in a minority of patients and so its impact was not considered during subsequent analysis.

3.3 Sample collections, DNA extraction and quantification

Approximately 20 mL peripheral blood samples from each patient were collected in EDTA tubes. DNA was extracted using Maxwell® RSC Whole Blood DNA Kit (Promega, Madison, USA) according to the manufacturer's instructions. Quality and concentration of DNA were assessed with NanoDrop spectrophotometers and Qubit 2.0 fluorimeter (Qubit dsDNA HS Assay Kit). DNA samples passing purity control were stored for downstream NGS experiment.

3.4 Library preparations, sequencing and data analysis

The Myeloid Solution Panel by SOPHiA Genetics is based on hybridization-capture chemistry and allows analysis of 30 genes (Fig. 1). At least 200 ng of pure DNA is used for the library preparation. DNA is first enzymatically fragmented and then end-repaired and A-tailed. DNA is then ligated with dual indexes adapters for sample. After these initial steps, post-ligation clean-up and dual-size selection with the use of magnetic beads are performed to remove non-bound adapters and select DNA fragments with a size distribution between 300bp and 700bp (~400 bp). After PCR amplification, the libraries are cleaned and quantified. Libraries' concentrations and quality control were assessed using Qubit and Agilent 2100 Bioanalyzer, as electrophoretic assay. After pooling the libraries in a single reaction, specific probes provided by Sophia Genetics are essential to capture the regions of interest. After hybridization and capture steps, streptavidin beads are used to purify the targets. Furthermore, a post-capture amplification is required. At the end of this PCR, the pooled libraries are processed to a new quantity and quality control. The pair-end sequencing of the pooled libraries is performed on the MiSeq Instrument (Illumina, San Diego, CA, USA) using V2 flow cell chemistry (2x251 cycles) with a PhiX library used as a sequencing control. Generated FASTQ files are uploaded on SOPHiA DDM® platform which allows detection, annotation, and pre-classification of mutations. Variants were checked in COSMIC [77], ClinVar [78], dbSNP [79] databases to select clinically relevant mutations; systematic literature review was performed to identify any possible novel variants.

According to American college of medical genetics and genomics (ACMG)/AMP-ASCOCAP guidelines [80,81], we selected only pathogenic/likely pathogenic variants with a variant allele frequency (VAF) around 5% or higher for subsequent prognostic analysis purpose. Variants detected below these thresholds or variants of unknown significance (C) were excluded from the analysis of clinical utility.

All driver mutations (*JAK2* V617F, *CALR* or *MPL*) were also detected with standards methods (qualitative PCR and digital droplet PCR for *JAK2* V617F, fragment analysis by capillary electrophoresis and Sanger Sequencing for *CALR* mutations and Sanger sequencing for *MPL*).

3.5 Statistical analysis

Continuous variables were expressed by the median and interquartile range (IQR), while categorical variables were summarized by number and percentage. Differences concerning categorical variables were estimated using the chi-square test or the Fisher exact test and the Mann-Whitney U test for continuous variables. OS was calculated from the diagnosis to death from any cause or the date of the last follow-up. EFS was instead calculated from the diagnosis to the date of an event (thrombosis, treatment failure, BP/AP, death) whichever comes first or the date of the last follow-up, censoring patients who underwent allo-HSCT. TTF was calculated for patients treated with ruxolitinib from the start of therapy to the date of resistance/loss of response or progression to AP/BP or death. Patients who were event free at the time of analysis were censored at the date of the last follow-up. Probabilities of OS, EFS and TTF were estimated using the Kaplan-Meier analysis and compared using the log-rank test. All p values <0.05 have been considered statistically significant. All statistical analyses were performed using the IBM SPSS Statistics, version 28.

4. Results

4.1 Validation runs

All the runs succeeded and completed the sequencing. In accordance with the manufacturer's instructions, all meet the expected ranges for quality assessment of data except for average percentage of target region with coverage ≥1000x for Run_1 which is below the expected range (92.6% vs expected values 95-99%), but still within the acceptable quality for variant calling and other downstream analyses. High quality of the validation runs was assessed with a sensitivity, specificity, accuracy, and a precision of 100%. Repeatability and reproducibility reached 99.98% and 99.99% respectively.

4.2 Baseline patients' characteristics

The baseline characteristics of patients at diagnosis are illustrated in Table 5. The whole cohort included 68 patients. Thirty-nine patients (57%) were male. Forty-six (68%) patients had PMF and 22 (32%) SMF (73% post-TE, 27% post-PV). For these latter, the median time from PV or TE diagnosis to MF evolution was 13.8 years (IQR 7.2-16.4). Among PMF, 17 (37%) had pre-PMF and 29 (63%) an overt type. The median age at diagnosis was 53 years (IQR 42-59). Median Hb, white blood cell count (WBC) and platelet count values for the entire cohort were 13.7 g/dL (IQR 11.6-13.7), 7.5 x 10^9/L (IQR 6.2-10.2) and 523 x 10^9/L (IQR 257.3-750), respectively. Median LDH level was 287 U/L (IQR 225-417). Karyotype was available and normal in 11 (16%) patients at diagnosis. Overall, a grade 2 or higher bone marrow (BM) fibrosis was observed in 46 (68%) patients. Thirty-seven percent of patients had disease-related symptoms. Most patients (60%) had splenomegaly and 22 out of 41 (54%) patients had a palpable splenomegaly ≥5 cm below the left costal margin (BLCM). At time of diagnosis, 8 (12%) patients had a previous history of thrombosis (4 splanchnic venous

thrombosis, 2 lower-limb deep vein thrombosis and 2 arterial thrombosis). According to the IPSS survival risk distribution at diagnosis in PMF, 32 (70%) patients were classified as low risk, 9 (20%) as intermediate-1, 3 (6%) as intermediate-2 and 2 (4%) as high (Table 6). In SMF, the MYSEC-PM risk distribution identified 7 (32%) patients as low risk, 13 (59%) as intermediate-1 and 2 (9%) as intermediate-2 (Table 6). Median follow-up was of 25 months (IQR 19.1–31.4) for the entire cohort.

4.3 NGS results

Driver mutations were distributed as follows: 35 (52%) *JAK2* V617F, 19 (28%) *CALR*, 3 (4.4%; 2 W515L and 1 W505N) *MPL* and a patient carried a double mutation (*JAK2* V617F/*MPL* W515Ki). Ten (15%) patients have been identified as triple negative (TN) cases, with no canonical driver mutation. Frequencies of driver mutations are depicted in Table 7. Type 1/type 1-like and type 2/type 2-like *CALR* mutations were found in 9 (47%) and 8 (42%) patients, respectively. Other complex *CALR* mutations were present in 2 (11%) patients which had not yet been published in the COSMIC dataset. The mutation c.1149_1154delinsTCCTTGTC is described in literature [82] whilst the mutation *CALR* c.1135_1144delinsCCTCCTCTTTGT could be a possible novel variant. Median VAF of driver mutations were 28.4% (IQR 14.9%-49.3%) for *JAK2* V617F, 38.1% (IQR 29.8%-41.5%) for *CALR*, and 42.1% (29.8%-68.7%) for *MPL* gene, without any significant differences in allele burden distribution (p=0.092). Aside from driver mutations, 72 non-driver mutations categorized as pathogenic (A, n=35, 49%) and likely pathogenic (B, n=37, 51%) were detected in 22/30 (73%) genes , whereas no mutations were identified in *ABL1, BRAF, IDH1/2, KIT, KRAS, NPM1* and *PTPN11* genes (Fig. 2). In detail, 53 (74%) were identified as single nucleotide variants (SNVs) and 19 (26%) were insertion/deletions (INDELS). Missense mutations (61%) are the most common protein-coding mutation found (Fig.3). The median number of additional mutations was 1 per patient (IQR 0-2), ranging from 0 to 6 (Table 7). Twenty-nine (43%) patients did not harbour any additional

mutations classified as A/B with a VAF ≥ 5%. We found 27 low variant fraction mutations that need a further validation and so they were not included for downstream analysis. Sixty percent of TN had at least one non-driver mutation, thus determining the clonal nature of the disease. The number of patients carrying a variant was illustrated in Table 8. The most frequent mutated genes were *TET2* (n=14, 20%), *ASXL1* (n=11, 16%) and *DNMT3A* (n=7, 10%). Seventeen patients (25%) carried at least 2 non-driver mutations and they were older [56 years (IQR 47-60) vs 48 (IQR 47-60), p=0.013), had significant higher level of monocytes [0.5 x 10^9/L (IQR 0.3-0.6) vs 0.3 x 10^9/L (IQR 0.2-0.4), p<0.001] and LDH [415 U/L (IQR 250-574) vs 255 U/L (IQR 220-321), p=0.001] at diagnosis than patients with less than 2 additional mutations. In addition, a slight significance was found for patients with ≥2 additional mutations who were more likely to have symptoms at diagnosis than patients with less than 2 mutations (88% vs 58%, p=0.042). Median values of spleen longitudinal diameter by abdomen ultrasound (p=0.035), monocytes (p<0.001), lymphocytes (p=0.015), LDH (p<0.001) were significant higher in *ASXL1* mutated patients compared to *ASXL1* wt, as showed in Table 9. *DNMT3A* mutations were instead associated with higher amount of WBC (p=0.043), neutrophils (p=0.047) and platelets (p=0.032) (Table 10). Median age at diagnosis was significantly higher for patients who carried *TET2* mutations [59 years (IQR 53-62) vs *TET2* wt 50 (IQR 40-57), p=0.009]. No other correlations were found between mutational profile and disease or patients characteristics. Overall, 13 (19%) patients had at least 1 HMR mutation; 3 (4%) patients had ≥2 HMR mutations. The majority of patients with HMR mutations had PMF (62% vs SMF 38%) and at least grade 2 BM fibrosis (69% vs grade 1, 31%), without reaching a statistical significance. The rate of symptomatic patients among HMR profile group was significantly higher than patients without HMR profile (69% vs 29%, p=0.011). Five out of 13 (38.5%) patients with HMR profile belonged to the low risk and intermediate-1 risk categories each, 1 (8%) to the intermediate-2 and 2 (15%) to the high-risk categories according to IPSS/MYSEC-PM.

4.4 Comparison according to driver mutations

Stratifying patients by driver mutation, those harboring *JAK2* V617F mutation showed a trend towards significance to be older at diagnosis [54 (IQR 44-59) vs 52 years (IQR 40-57), p=0.060) and to have higher median Hb level [14.3 g/dL (IQR 12.4-15.8) vs 12.5 (IQR 11.8-14.3), p=0.050) compared to *CALR/MPL* mutated or TN patients. The *JAK2* V617F driver mutation was significantly associated with higher WBC [8.2 x 10^9/L (IQR 6.6-14.5) vs 7.1 10^9/L (IQR 6.1-8.2), p=0.010] and neutrophils amount [5.8 x 10^9/L (IQR 4.7-8.8) vs 4.5 x 10^9/L (IQR 3.7-5.4), p=0.002] than *JAK2* wt. No significant correlations were found between *JAK2* V617F and the type or the number of non-driver mutations, grade of fibrosis or other clinical and biological features. CALR mutated subgroup had significantly higher platelet count [725x10^9/L (IQR 501.5-1.063) vs 484.0 x10^9/L (IQR 237.0-654.0), p<0.001] and LDH value [418 U/L (IQR 264-653) vs 252 U/L (IQR 220-320), p=0.002] than *CALR* wt patients]. In addition, *CALR* mutated patients were more likely to have mutations in *ASXL1* gene compared to those with *JAK2 V617F* (32% vs 8%, p=0.030, Fig. 4). TN patients showed instead significantly lower median value of platelets [173 x10^9/L (IQR 107-422) vs JAK2/CALR 535 x10^9/L (IQR 378-833), p=0.002].

4.5 Differences between PMF and SMF

As illustrated in Table 5, SMF patients were older than PMF at diagnosis [57 (IQR 46-61) vs 50 years (IQR 40-56), p=0.027); furthermore, SMF patients showed significant superior value of LDH at baseline [329 (IQR 289-494) vs 250 U/L (IQR 220-374), p=0.004) and were more likely to have disease-related symptoms [SMF 59% vs PMF 26%, p=0.015) and splenomegaly (SMF 82% vs PMF 50%, p=0.017). Among patients with JAK2 V617F, the allele burden was significantly higher for SMF compared to PMF [VAF 43% (IQR 39.5-41.7) vs 21.4% (IQR 9.4-39.4), p=0.023, Table 7]. No other differences were seen regarding the type and the number of non-driver mutations (Tables 7-8).

4.6 MIPSS70 assessment and risk-adapted therapy

The application of MIPSS70 identified 22 patients, who were previously categorized as low risk according to IPSS/MYSEC-PM, in intermediate risk and allocated 3 patients, who were previously considered as intermediate-1 risk (n=2) and intermediate-2 risk (n=1), in high-risk category, deeply influencing the treatment strategy. Globally, according to MIPSS70 score, patients were divided into low risk (n=18, 26%), intermediate (n=45, 66%) and high risk (n=5, 7%) (Table 6, Fig. 5). In particular, carrying a HMR profile was responsible for shifting risk category in 7 patients (10% of all population studied).

Graphic representation of treatment strategy according to MIPSS70 risk categories is illustrated in Fig. 6.

Allogeneic HSCT was recommended in all 5 high risk patients (4 with HMR profile), including those who were previously stratified as intermediate-1 or intermediate-2 risk: 2 were rapidly performed, 2 were waiting at the last follow-up and 1 patient lost his eligibility due to SARS-CoV2 infection and then died in progression. HMR profile was determinant in proposing transplant choice in 3 out 9 (33%) patients categorized as intermediate risk, but only one underwent transplant whereas a patient was referred to other Centre and the other one rapidly progressed to BC concomitantly with a pulmonary infection. For the other patients with HMR mutation status in intermediate risk, research of the most suitable related or unrelated HLA donor has been started; they were monitored more closely and were stable at last follow-up.

Overall, 28 (41%) patients did not require any treatments and were on clinical treatment-free follow-up; nobody of them carried HMR profile. Thirty-nine (57%) patients needed a treatment: 21 (54%) ruxolitinib, 3 (8%) interferon, 14 (36%) hydroxyurea, 1 (2%) allogeneic HSCT without a bridge

therapy. Considering all observation period, 7 patients underwent allogeneic HSCT. Overall, 4 out of 68 (6%) patients died: 2 for BC, 1 for transplant complication and 1 for SARS-CoV-2 infection in AP.

4.7 Ruxolitinib treatment and time-to-treatment failure

Focusing on patients who received ruxolitinib, the main characteristics are showed in Table 11. Eleven out of 21 (53%) were male; the median age at the start of ruxolitinib was 57 years (IQR 44-59). All but 2 patients had disease-related symptoms; all had a palpable splenomegaly and the median value of spleen longitudinal diameter by ultrasound scanning was 20 cm (IQR 18-21). Eight (38%) carried at least one of HMR mutations. The median values of haematological parameters and starting dose of ruxolitinib are also depicted in Table 11. The majority of patients (76%) had a rapid spleen response and improvement of MF-related symptoms at 3-month assessment of response, regardless of baseline mutational profile. Five (24%) patients were primary refractory and 2 of them had *ASXL1* mutation. In summary, after a median time of 12 months (IQR 7-17.5), 11 (52%) patients definitely stopped ruxolitinib. In particular, 6 (55%) patients underwent allogeneic HSCT, 2 (18%) were enrolled in a clinical-trial and 3 (27%) progressed to BC/AP. Two of these latter had *RUNX1* mutation. Median TTF was 19.1 months (95%CI 9.6-28.6) (Fig. 7). Age (p=0.292), type of MF (p=0.114), IPSS (p=0.924), DIPSS (p=0.720), MYSEC-PM (p=0.525), grade of fibrosis (p=0.523) and peripheral blood blasts (p=0.662) did not influence TTF. High risk category according to MIPSS70 showed significantly shorter TTF than patients at intermediate risk [median TTF 6.5 months (95%CI 0.8-12.1) vs not reached, p=0.005, Fig. 8]. Patients with ≥2 additional mutations seemed to have shorter TTF even if these findings did not reach a statistical significance [median TTF 19 months vs <2 mutations, median TTF not reached, p=0.106, Fig. 9), while HMR signature did not impact TTF in our cohort (p=0.491).

4.8 Overall Survival

Median OS for the whole cohort was not reached; 1-year, 2-year and 3-year survival probabilities were 98.3%, 95.6% and 91.1% (Fig. 10), respectively. Age ≥60 years was associated with inferior survival [1-year OS 88.7% vs 100%, p=0.006, Fig.11]. We found statistically significant differences between low and intermediate-2 risk categories according to IPSS [3-year OS low risk 100% vs intermediate-2 87.5%, p=0.017 Fig 12A]. The other prognostic clinical scores did not have an impact on survival (DIPSS, p=0.429; MYSEC-PM for SMF, p=0.368; Fig. 12B-C). On the contrary, high risk category according to MIPSS70 prognostic score was associated with a significant reduction of 3-year survival probability [50% vs low risk 100% vs intermediate risk 97.3%, p=0.034; low vs intermediate, p=0.410; intermediate vs high, p=0.005; low vs high, p=0.029 Fig. 12D]; this result was more evident when comparing high vs low/intermediate risk groups [3-year OS 50% vs 98.1%, respectively (p=0.022)]. Constitutional symptoms at diagnosis were associated with inferior 3-year OS [76.6% vs 100% in asymptomatic patients, p=0.021 Fig. 13]. Type of MF (p=0.618, Fig. 14), clinical stage of PMF (0.339), driver mutations (p=0.470, Fig. 15), splenomegaly (p=0.414) and grade of fibrosis (p=0.278) did not influence outcome in terms of OS. Harboring at least one HMR mutation or ≥2 non-driver mutations negatively influenced survival [≥1 HMR median OS 49.1 months (95%CI 21.3-77.6) vs no HMR median OS not reached. p=0.032, Fig. 16; ≥2 non-driver mutations 3-year OS 78.7% vs <2 non-driver mutations 100%, p= 0.025, Fig. 17]. Patients carrying *ASXL1* mutations showed a trend for worse OS (p=0.080, Fig. 18), while *RUNX1* mutated patients had significant inferior median OS [*RUNX1* mut 11.6 months vs *RUNX1* wt not reached, p=0.002, Fig. 19]. Separately analysing the PMF and SMF groups, HMR profile still maintained its prognostic significance for PMF (p<0.001, Fig. 20A), but not for SMF patients (p=0.906, Fig. 20B). In addition, *ASXL1* mutations negatively impacted OS among PMF patients [ASXL1 mut 2-year OS 75% vs ASXL1 wt 97% , p=0.019 Fig. 21A], but not in SMF group (p=0.480, Fig. 21B).

4.9 Event-Free Survival

Median EFS was not reached. Estimates probability of EFS at 6 months, 1 year and 3 years were 97%, 95.4% and 76.7%, respectively (Fig. 22), without any differences between PMF and SMF groups (p=0.928). Patients stratified as low risk showed significant advantage in terms of median EFS than those at intermediate-1, intermediate-2 or high risk according to IPSS score [median EFS not reached vs 24.1 months (95%CI 2.4-88.1), p<0.001 Fig. 23]. Among SMF, MYSEC-PM score did not have any impact on EFS (p=0.166). Conversely, estimated 3-year EFS was significantly inferior for high-risk patients by MIPSS70 model [26.1% vs 80% intermediate risk vs 100% low risk, p=0.005, Fig. 24]. Presence of disease-related symptoms (3-year EFS 59.5% vs no symptoms 100%, p<0.001, Fig. 25) and palpable splenomegaly [yes, median EFS 36 months (95%CI 24.4-47) vs no, median EFS not reached, p=0.020, Fig. 26) were significantly associated with poorer outcomes. Median EFS was negatively influenced by HMR profile [median EFS 36.1 months (95%CI 23.3-48.7) vs median EFS not reached, p=0.006, Fig. 27]. Among all genes mutated, only *ASXL1* mutations had a negative prognostic impact [median EFS 31 months vs ASXL1 wt, median EFS not reached, p=0.012 Fig. 28]. In addition, EFS was adversely affected by presence of at least 2 non-driver mutations [median EFS 36 months (95%CI 23.4-46) vs median EFS not reached for patients harbouring <2 non-driver mutations, p=0.035, Fig. 29]. Molecular impact on EFS still remained significant for *ASXL1* mutational status (p<0.001, Fig. 30 A), presence of HMR profile (p<0.001, Fig. 31 A) and additional non-driver mutations (p=0.004, Fig. 32 A) in PMF but not in SMF group (p values= 0.287, 0.906, 0.694, respectively Fig. 30 B, 31 B, 32 B).

5. Discussion

MF is a clinically and genetically complex hematologic disorder. Driver mutations have a crucial role in pathogenesis. Survival is highly variable and different clinical prognostic scores have been routinely used in clinical practice [1]. Technologic advancements in DNA sequencing have increased the discover of additional mutations that may impact prognostication and, therefore, treatment strategies. In this project, we aimed to use NGS technology in order to refine prognostic risk and treatment decisions in MF patients in a real-life setting. We also aimed to correlate biological insights with clinical findings and discover possible predictors of ruxolitinib resistance. Our cohort consisted of 68 MF patients and most of them had PMF, splenomegaly and disease-related symptoms. All patients were ≤65 years old and were potentially suitable for allogeneic HSCT.

In our cohort driver mutations distribution perfectly reflect the epidemiology of the disease as *JAK2* V617F was the most frequent among them. We confirmed also the mutually exclusively manner of driver mutations [8]. Indeed, only one patient concomitantly carried two driver mutations in *JAK2* and *MPL* genes, respectively. In clinical practice, searching for driver mutations is mainly a multi-step process and so, the co-occurrence of driver mutations is probably underestimated in real-life setting. We retained that NGS tools allowing to target in multiple genes at the same time with higher sensitivity compared to traditional methods, could help to define the real incidence of co-existing driver mutations and the true relevance of them on outcome. In addition, NGS is useful to support diagnosis of MF, demonstrating clonal somatic mutations in 60% TN cases of our real-life study. Evaluating the clinical features according to the type of driver mutations, as previously reported in the literature [8,83], *JAK2* V617F is associated with leucocytosis and a trend towards significance for higher Hb value and age at diagnosis in our cohort, even if we included in the project only patients aged 65 or less. Higher platelets count was found among *CALR* mutated patients

whereas TN cases showed significant inferior platelets count at baseline, resulting in line with literature evidence [8,84]. Published data reported that *CALR* type-1 mutated patients, particularly in PMF, have superior survival to those with other mutations, whereas TN-MF patients have the worst outcome [18,19]. Conversely, we did not identify any prognostic impact of driver mutations in terms of survival, probably due to the small sample size and relatively short observation period. We discovered 72 non-driver mutations with a median of 1 mutation per patient. Several genes of the panel not showed mutations as some of them are more likely impaired in AP/BC or de-novo AML, as *PTPN11*, *NPM1* [8,25].

We found several differences among clinical characteristics according to the type and the number of additional mutations.

In line with the literature [8], the number of additional mutations and *TET2* mutations are associated with older age; additionally, having 2 or more non-driver mutations was associated with more pronounced disease-features, as splenomegaly, symptoms burden, LDH, monocytes and lymphocytes count, presumably due to more active disease. No specific phenotypic signature related to *DNMT3A* was described in literature [1,8] but we showed that *DNMT3A* mutated patients have some hyperproliferative characteristics, as higher number of neutrophils and platelets than *DNMT3A* wt.

In our cohort, *ASXL1* mutated patients carried distinct high-risk phenotypic features, including higher value of LDH, monocytes, lymphocytes, spleen longitudinal diameter and the presence of constitutional symptoms, suggesting higher risk disease than *ASXL1* wt patients. Several other experiences reported high-risk disease characteristics for *ASXL1* mutated MF, irrespective of their driver mutations [8, 32, 85]. Guglielmelli et al. [32] found similar associations in their multicentre analysis that included 330 PMF patients. In their study, *ASXL1* mutation was associated with older

age, male sex, higher leukocyte count, lower Hb level, fewer platelets, more peripheral blasts, BM fibrosis grade ≥2, constitutional symptoms and transfusion dependence compared to *ASXL1* wt cohort. Interestingly, the critical role of *ASXL1* mutations in inducing monocyte/macrophage and neoplastic monocyte-derived fibrocyte differentiation was recently investigated by Shi et al. [86]. The authors showed that *ASXL1* mutation in hematopoietic stem and progenitor cells (HSPCs) leads to upregulation of EGR1, a polycomb group target gene. EGR1 increase the commitment of HSPCs to monocyte/macrophage lineage and, through the activation of TNFα, the differentiation of monocytes to fibrocytes, consequently enhancing inflammation and BM fibrosis [86]. These findings could partially explain the mechanism by which aberrations in *ASXL1* gene are associated with more aggressive features in MF and could also represent future horizons of targeted therapy [87.]. In our experience, we found no correlations between ASXL1 mutations and grade of fibrosis but we could speculatively hypothesize that the association between *ASXL1* and the amount of monocytes may indirectly influence grade of fibrosis.

From a clinical point of view, the prognostic role of *ASXL1* and the inclusion of other genes in HMR category are recently questioned. The French Intergroup of Myeloproliferative Neoplasms (FIM) [33] evaluated the mutational landscape of 305 PMF and 174 SMF, showing the lack of survival prognostic significance for *ASXL1* isolated mutations (without TP53 or other high-risk mutations) and proposing a new 4-tier model "NGS" to assess prognostic risk of MF patients. Moreover, the authors demonstrated that the combination of their genomic classification combined with IPSS or MYSEC-PM have better prognostic performance than MIPSS70, shedding light on *TP53*, *U2AF1*, *CBL*, *NRAS*, and *KRAS* as new possible high risk genes [33]. Guglielmelli et al. [32] analysed 530 MF patients (330 PMF and 193 SMF) confirming the negative prognostic role of *ASXL1* in PMF, even in the absence of any co-occurring high-risk mutations, but not in SMF; the authors established that MIPSS70 had the best predictive performance in PMF whereas combination of the model proposed

by Luque Paz [33] and MYSEC-PM better performed in predictive deaths in SMF, strengthening the concept that PMF and SMF are 2 different biological entities [32].

In our prospective experience, we confirmed the prognostic role of IPSS in PMF but not of MYSEC-PM in SMF and, contrary to the literature [7, 89, 90], we did not find any differences among PMF and SMF, according to mutational profile, probably because of the low number of SMF patients. Of note, we found that SMF were older and had more symptoms, splenomegaly and higher VAF of *JAK2* V617F compared to PMF, as previously reported [89, 90].

Real-life successful validation of MIPSS70, originally conceived for PMF, was carried out in 218 PMF patients, from the GEMFIN multicentre database [91]; Hernández-Sánchez et al. [91] demonstrated that the score was able to stratify patients in the 3 categories (low 13%, intermediate 35% and high risk 52%) with significant differences in OS. In their study, *ASXL1* was mutated in 27% of patients, but it did not impact survival whereas *SRSF2*, *CBL*, *SETBP1* and *KRAS* mutations were instead associated with worse outcome. The authors did not report the possible role of the non-driver mutations' number.

In our study, MIPSS70 risk patients distributions were 26% in low, 66% in intermediate and 7% in high risk; globally, 37% of patients changed their risk category compared to traditional clinical prognostic score and the identification of HMR profile was determinant in shifting category in 10% of our population studied, deeply influenced treatment strategy. Indeed, in our cohort allogeneic HSCT was proposed in all high-risk patients and in the 33% of patients carrying HMR at intermediate risk. Careful monitoring and prompt research for the eventually most suitable donor was started for the other individuals with HMR mutations.

HMR profile or having at least 2 additional mutations negatively influenced overall outcome, both in terms of OS and EFS. *ASXL1* mutated patients showed a tendency to have inferior OS but

significant shorter EFS compared to *ASXL1* wt. These findings were confirmed only for PMF subgroup of our cohort. IPSS but not MYSEC-PM impacted outcome, probably due to relatively low number of SMF. High risk of MIPSS, considering the whole population studied, showed inferior OS and EFS than low and intermediate risks. Moreover, *RUNX1* mutated patients had significantly shorter survival than *RUNX1* wt. Although *RUNX1* is not included in HMR genes, other experiences in literature showed its association with shorter OS and leukemia-free survival [8,92], suggesting that addressed further investigations are needed.

Controversies still exists regarding the possible impact of mutational status on outcome in JAK1-2 inhibitor treated patients. To address this issue, we analysed 21 patients who received ruxolitinib confirming its clinical benefit (spleen reduction and symptoms improvements) irrespective of biological findings, including HMR signature that was present in 38% of this subgroup. These findings were consistent with analysis of COMFORT-II [67] by which ruxolitinib response rates were similar across different mutation profiles.

 In contrast to the findings of the COMFORT-II study [67], the number of mutations was inversely correlated with spleen response and 3 or more of mutations among *ASXL1*, *EZH2*, *IDH1*, or *IDH2* genes were predictor of short time to treatment discontinuation in a monocentric study reported by Patel et al. among 95 MF patients [93].

England et al. [94] showed that baseline *CBL* mutations, but not the number of mutations, had a significant predictive value for JAK-inhibitors failure in their retrospective study involving 113 MF patients followed at Princess Margaret Cancer Centre. They also found that *RAS* pathway genes and HMR genes were the most common class of emergent mutations at the time of JAK-inhibitors failure [94].

Recently, at the 64th American Society of Hematology Annual Meeting and Exposition 2022 (ASH 2022), several studies have been shared aiming to identify possible molecular predictors of response in JAK-inhibitors treated patients.

Gangat et al. [95] retrospectively compared long-term treatment outcomes of PMF and SMF patients enrolled on ruxolitinib (NCT00509899), fedratinib (NCT00631462, NCT01420770), momelotinib (NCT00935987, NCT01236638), or BMS-911543 (NCT01236352) clinical trials at the Mayo Clinic, revealing that fedratinib use and *ASXL1* wt status predicted spleen response, while *SRSF2* mutations were associated with anemia response.

In an Italian study [96] including 171 consecutive patients with a diagnosis of PMF/SMF treated with ruxolitinib, the rate of non-responders (NR) and responders patients were 54.4% and 45.6%, respectively, after a median follow-up from ruxolitinib start of 3.5 years. Among responders, 57.7% lost response after a median time of 22.7 months and 43.3% maintained response. Response outcome was influenced by ruxolitinib dose, which was significantly higher in patients who maintained response compared to those who lost it, and also by the baseline molecular variables. Isolated *CBL* and *U2AF1* mutation were exclusively found in non-responders patients and HMR status was less frequently carried by patients with a sustained response. Furthermore, at least one *RAS* pathway mutated gene (*CBL, NRAS, KRAS, PTPN11*) was more likely found in non-responders patients [96].

Maslah et al. [97] demonstrated that ruxolitinib treatment was independently associated with *RAS* mutations acquisition in a longitudinal molecular evaluation of 73 MF patients. *In vitro* experiments also confirmed the direct effect of ruxolitinib-induced JAK inhibition on *RAS* clonal selection. Despite a validation of these results is certainly needed, this study defines an important oncogenic

mechanism and suggests that screening for *RAS* mutated clones before ruxolitinib treatment could allow a tailored alternative treatment and avoid clones expansion in *RAS* mutated patients.

In our cohort 5 patients carried at least one RAS pathway mutated gene. Two of them were treated with ruxolitinib: a patient lost ruxolitinib response and died for BC while the other one underwent allogeneic HSCT and was alive at the last follow-up. The other patients received other treatments (IFN, HU) and were alive at the last follow-up.

In our experience patients with at least 2 non-driver mutations at baseline had shorter TTF, albeit not reaching significance.

Additionally, in our cohort MIPSS-70 high-risk patients had shorter TTF compared to patients at intermediate- risk, whilst IPSS/DIPSS/MYSEC-PM stratification had no impact on TTF.

Although the low rate of patients in high-risk category, we confirmed that identification of these patients, through the integration of clinical and biological features, is crucial for early discussing the role of ruxolitinib versus clinical trial or allogeneic HSCT when feasible.

In conclusion, our monocentric and prospective study demonstrates the feasibility of integrating biological findings using NGS tool in the workflow of MF patients. Our study is an attempt to translate molecular findings into a more accurate risk assessment and risk-adapted management of MF patients in clinical practice. Urgent unmet needs remain the identification of predictive factors to ruxolitinib response and a more specific risk model for SMF patients, paving the way for novel target therapy.

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7. Appendix

Table 1. 2016 revised World Health Organization (WHO) diagnostic criteria for primary myelofibrosis

	Primary myelofibrosis (prefibrotic) (Diagnosis requires meeting all 3 major criteria and 1 minor criterion)	Primary myelofibrosis (overtly fibrotic) (Diagnosis requires meeting all 3 major criteria and 1 minor criterion)
Major criteria		
	1 Typical megakaryocyte changes, accompanied by ≤grade 1 reticulin/collagen fibrosis	1 Typical megakaryocyte changes, accompanied by ≥grade 2 reticulin/collagen fibrosis
	2. Presence of JAK2 CALR or MPL mutations, or presence of other clonal markers[a] or absence of evidence for reactive bone marrow fibrosis	2. Presence of JAK2 CALR or MPL mutations, or presence of other clonal markers[a] or absence of evidence for reactive bone marrow fibrosis
	3. Not meeting WHO criteria for other myeloid neoplasms	3. Not meeting WHO criteria for other myeloid neoplasms
Minor criteria		
	1. Anemia not otherwise explained 2. Leucocytosis ≥ 11×10^9/L 3. Palpable splenomegaly 4. Increased serum lactate dehydrogenase	1. Anemia not otherwise explained 2. Leucocytosis ≥ 11×10^9/L 3. Palpable splenomegaly 4. Increased serum lactate dehydrogenase 5. A leucoerythroblastic blood smear

Table adapted from Barbui T et al. *Blood Cancer J*. 2018;8(2):15.

Footnotes:

a. In the absence of any of the three major clonal mutations, the search for the most frequent accompanying mutations *(ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1*) are of help in determining the clonal nature of the disease.

Table 2. International Working Group for Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) recommended criteria for post-polycythemia vera (post-PV MF) and post-essential thrombocythemia myelofibrosis (post-ET MF)

	Post-polycythemia vera myelofibrosis (post-PV MF) (Diagnosis requires meeting both major criteria and at least 2 minor criteria)	Post-essential thrombocythemia myelofibrosis (post-ET MF) (Diagnosis requires meeting both major criteria and at least 2 minor criteria)
Major criteria		
	1. Prior documentation of WHO-defined PV	1. Prior documentation of WHO-defined ET
	2. Bone marrow fibrosis grade ≥ 2	2. Bone marrow fibrosis grade ≥ 2
Minor criteria		
	1. Anemia or loss of phlebotomy requirement	1. Anemia and ≥ 2g/dL decrease in hemoglobin level
	2. A leucoerythroblastic blood smear	2. A leucoerythroblastic blood smear
	3. Increasing splenomegaly	3. Increasing splenomegaly
	4. Development of constitutional symptoms	4. Development of constitutional symptoms
		5. Increased serum lactate dehydrogenase

Table adapted from Barosi G et al. *Leukemia*. 2008;22(2):437-438.

Table 3. Prognostic models for myelofibrosis

	Clinical Variables	Genetic variables	Points and Risk categories
IPSS		-	
	Age >65 years *(1 point)*		**0 Low**
	Presence of constitutional symptoms *(1 point)*		**1 Intermediate-1**
	Hemoglobin <10 g/dL *(1 point)*		**2 Intermediate-2**
	WBC count >25x10^9/L *(1 point)*		**≥3 High**
	Blood blasts ≥1% *(1 point)*		
DIPSS		-	
	Age >65 years *(1 point)*		**0 Low**
	Presence of constitutional symptoms *(1 point)*		**1-2 Intermediate-1**
	Hemoglobin <10 g/dL *(2 points)*		**3-4 Intermediate-2**
	WBC count >25x10^9/L *(1 point)*		**≥5 High**
	Blood blasts ≥1% *(1 point)*		
DIPSS-plus		-	
	Age >65 years *(1 point)*		**0 Low**
	Presence of constitutional symptoms *(1 point)*		**1 Intermediate-1**
	Hb <10 g/dL *(2 points)*		**2-3 Intermediate-2**
	WBC count >25x10^9/L *(1 point)*		**≥4 High**
	Blood blasts ≥1% *(1 point)*		
	RBC transfusion dependency *(1 point)*		
	Platelets <100 x 10^9/L *(1 point)*		
	Unfavourable karyotype* *(1 point)*		
MIPSS 70			
	Hb <10 g/dl *(1 point)*	One HMR mutation** *(1 point)*	**0-1 Low**
	WBC >25 x 10^9/L *(2 points)*	≥ 2 HMR mutations *(2 points)*	**2-4 Intermediate**
	Platelets <100 x 10^9/L *(2 points)*	Type 1/like CALR absent *(1 point)*	**≥5 High**
	Circulating blasts ≥2% *(1 point)*		
	Constitutional symptoms *(1 point)*		
	Bone marrow fibrosis grade ≥2 *(1 point)*		

MIPSS70+ version 2	Severe anemia (Hb <8 g/dL in women and <9 g/dL in men) *(2 points)* Moderate anemia (Hb 8–9.9 g/dL in women and 9–10.9 g/dL in men) *(1 point)* Circulating blasts ≥2% *(1 point)* Constitutional symptoms *(2 points)*	VHR karyotype *(4 points)* Unfavourable karyotype *(3 points)* ≥ 2 HMR mutations *(3 points)* One HMR mutation *(2 points)* Type 1/like CALR absent *(2 points)*	**0 Very low** **1–2 Low** **3–4 Intermediate** **5–8 High** **≥9 Very high**
GIPSS	-	VHR karyotype *(2 points)* Unfavourable karyotype *(1 point)* Type 1/like CALR absent *(1 point)* ASXL1 mutation *(1 point)* SRSF2 mutation *(1 point)* U2AF1Q157 mutation *(1 point)*	**0 Low** **1 Intermediate-1** **2 Intermediate-2** **≥3 High**
MYSEC-PM (for SMF)	Age at diagnosis *(0.15 per patient's year of age)* Hb <11 g/dL *(2 points)* Circulating blasts ≥3% *(2 points)* Platelets <150 x 10^9/L *(1 point)* Constitutional symptoms *(1 point)*	Type 1/like CALR absent *(2 points)*	**<11 Low** **≥11 Intermediate-1** **≥14-<16 Intermediate-2** **≥16 High**

Abbreviation: Hb, hemoglobin; HMR, high molecular risk; RBC, red blood cells; VHR, very high risk; WBC, white blood cells count

Footnotes:

*Unfavorable karyotype: complex karyotype or sole or two abnormalities that include trisomy 8, 7/7q-, i(17q), 5/5q-, 12p-, inv(3), or 11q23 rearrangement.

** Presence of a mutation in any of the following genes: *ASXL1*, *EZH2*, *SRSF2* or *IDH1/2 (also U2AF1* Q157 for MIPSS70+ version 2)

*** VHR karyotype: single/multiple abnormalities of −7, i(17q), inv(3)/3q21, 12p−/12p11.2, 11q−/11q23, or other autosomal trisomies not including + 8/+9 (eg, +21, +19).

Table 4. Revised IWG-MRT and ELN response criteria for MF

Response categories	Required Criteria (for all response categories, benefit must last for ≥12 weeks to qualify as a response)
Complete remission (CR)	Bone marrow: Age-adjusted normocellularity; <5% blasts; ≤grade 1 MF and peripheral blood: Hb ≥10 g/dL and <UNL; neutrophil count ≥1 x 10^9/L and <UNL; platelet count ≥100 x 10^9/L and <UNL; <2% immature myeloid cells‡ and clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH
Partial remission (PR)	Peripheral blood: Hb ≥10 g/dL and <UNL; neutrophil count ≥1 x 10^9/L and <UNL; platelet count ≥100 x 10^9/L and <UNL; <2% immature myeloid cells‡ and Clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH or Bone marrow: Age-adjusted normocellularity; <5% blasts; ≤grade 1 MF and peripheral blood: Hb ≥8.5 but <10 g/dL and <UNL; neutrophil count ≥1 x 10^9/L and <UNL; platelet count ≥50, but <100 x 10^9/L and <UNL; <2% immature myeloid cells‡ and Clinical: Resolution of disease symptoms; spleen and liver not palpable; no evidence of EMH
Clinical improvement (CI)	The achievement of anemia, spleen or symptoms response without progressive disease or increase in severity of anemia, thrombocytopenia or neutropenia§
Anemia response	Transfusion-independent patients: a ≥2 g/dL increase in Hb level° Transfusion-dependent patients: becoming transfusion-independent*
Spleen response #	A baseline splenomegaly that is palpable at 5-10 cm, below the LCM, becomes not palpable** or A baseline splenomegaly that is palpable at >10 cm, below the LCM, decreases by ≥50%** A baseline splenomegaly that is palpable at <5 cm, below the LCM, is not eligible for spleen response A spleen response requires confirmation by MRI or CT showing ≥35% spleen volume reduction
Symptoms response	A ≥50% reduction in the MPN-SAF TSS
Progressive disease*	Appearance of a new splenomegaly that is palpable at least 5 cm below the LCM or A ≥100% increase in palpable distance, below LCM, for baseline splenomegaly of 5-10 cm or A 50% increase in palpable distance, below LCM, for baseline splenomegaly of >10 cm or Leukemic transformation confirmed by a bone marrow blast count of ≥20% or

	A peripheral blood blast content of ≥20% associated with an absolute blast count of ≥1 x 10^9/L that lasts for at least 2 weeks
Stable disease	Belonging to none of the above listed response categories
Relapse	No longer meeting criteria for at least CI after achieving CR, PR, or CI, or Loss of anemia response persisting for at least 1 month or Loss of spleen response persisting for at least 1 month

Recommendations for assessing treatment-induced cytogenetic and molecular changes	
Cytogenetic remission	At least 10 metaphases must be analyzed for cytogenetic response evaluation and requires confirmation by repeat testing within 6 months window CR: eradication of a pre-existing abnormality PR: ≥50% reduction in abnormal metaphases (partial response applies only to patients with at least ten abnormal metaphases at baseline)
Molecular remission	Molecular response evaluation must be analyzed in peripheral blood granulocytes and requires confirmation by repeat testing within 6 months window CR: Eradication of a pre-existing abnormality PR: ≥50% decrease in allele burden (partial response applies only to patients with at least 20% mutant allele burden at baseline)
Cytogenetic/molecular relapse	Re-emergence of a pre-existing cytogenetic or molecular abnormality that is confirmed by repeat testing

Table adapted from Tefferi A et al. *Blood*. 2013;122(8):1395-1398

Abbreviations: CT, computed tomography; EMH, extramedullary hematopoiesis; Hb, hemoglobin; LCM, left costal margin; MRI, Magnetic Resonance Imaging; UNL, upper normal limit

Footnotes:

‡ Immature myeloid cells constitute blasts 1 promyelocytes 1 myelocytes 1 metamyelocytes 1 nucleated red blood cells. In splenectomized patients, <5% immature myeloid cells is allowed.

§ Increase in severity of anemia constitutes the occurrence of new transfusion dependency or a ≥2 g/dL decrease in Hb level from pre-treatment baseline that lasts for at least 12 weeks. Increase in severity of thrombocytopenia or neutropenia is defined as a 2-grade decline, from pre-treatment baseline, in platelet count or absolute neutrophil count, according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. In addition, assignment to CI requires a minimum platelet count of ≥25 000 x 10^9/L and absolute neutrophil count of ≥0.5 x 10^9/L.

° Applicable only to patients with baseline Hb of <10 g/dL. In patients not meeting the strict criteria for transfusion dependency at the time of study enrollment, but have received transfusions within the previous month, the pretransfusion Hb level should be used as the baseline.

* Transfusion dependency before study enrollment is defined as transfusions of at least 6 units of packed red blood cells (PRBC), in the 12 weeks prior to study enrollment, for a Hb level of <8.5 g/dL, in the absence of bleeding or treatment-induced anemia. In addition, the most recent transfusion episode must have occurred in the 28 days prior to study enrollment. Response in transfusion-dependent patients requires absence of any transfusions during any consecutive "rolling" 12-week interval during the treatment phase, capped by Hb level of ≥8.5 g/dL.

\# In splenectomized patients, palpable hepatomegaly is substituted with the same measurement strategy.

**Spleen or liver responses must be confirmed by imaging studies where a ≥35% reduction in spleen volume, as assessed by MRI or CT, is required. Furthermore, a ≥35% volume reduction in the spleen or liver, by MRI or CT, constitutes a response regardless of what is reported with physical examination.

***Progressive disease assignment for splenomegaly requires confirmation by MRI or CT showing a ≥25% increase in spleen volume from baseline. Baseline values for both physical examination and imaging studies refer to pre-treatment baseline and not to posttreatment measurements.

Table 5. Baseline patients' characteristics

Characteristics	Overall No. of patients=68	PMF No. of patients= 46	SMF No. of patients=22	*p* value
Gender, n (%)				
Male, n (%)	39 (57)	29 (63)	10 (45.5)	ns
Female, n (%)	29 (43)	17 (37)	12 (54.5)	
Type of PMF, n (%)				
Pre/Early -PMF	-	17 (37)	-	
Overt-PMF		29 (63)		-
Type of SMF, n (%)				
PV-MF	-	-	6 (9)	
TE-MF			16 (24)	-
Median age at diagnosis (IQR, years)	53 (42-59)	50 (40-56)	57 (46-61)	*0.027*
Hb, g/dL				
Median (IQR)	13.7 (11.6-15.0)	13.5 (11.7-15.0)	13.8 (11.4-14.8)	ns
WBC × 10^9/L				
Median (IQR)	7.5 (6.2-10.2)	7.5 (6.2-9.1)	7.8 (6.1-15.2)	ns
Neutrophils x 10^9/L				
Median (IQR)	5.1 (3.9-7.2)	5.1 (3.7-6.8)	5.6 (3.9-11.1)	ns
Monocytes x 10^9/L				
Median (IQR)	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0.4 (0.3-0.6)	ns
Lymphocytes x 10^9/L				
Median (IQR)	1.6 (1.1-2.0)	1.6 (1-1-2.1)	1.6 (1.1-1.9)	ns
Platelet count × 10^9/L	523	526	436	
Median (IQR)	(257-771)	(243-784)	(373-747)	ns
LDH, U/L	287	250	328	
Median (IQR)	(225-417)	(220-374)	(289-494)	*0.004*
Creatinine mg/dL				
Median (IQR)	0.8 (0.7-0.9)	0.8 (0.7-1.0)	1.2 (0.7-0.9)	ns

PB blast percentage Median (IQR)	0 (0-3)	0 (0-2)	0 (0-2)	ns
BM fibrosis grade, n (%)				
Grade 1	22 (32)	17 (37)	5 (22.7)	*0.036*
Grade 2	26 (38)	20 (43.5)	6 (27.3)	
Grade 3	20 (29)	9 (19.6)	11 (50)	
Previous history of thrombosis, n (%)				
Yes	8 (12)	4 (9)	4 (18)	ns
No	60 (88)	42 (91)	18 (82)	
Disease-related symptoms, n (%)				
Yes	25 (37)	12 (26)	13 (59)	*0.015*
No	43 (63)	34 (74)	9 (41)	
Splenomegaly, n (%)				
Yes	41 (60)	23 (50)	18 (82)	*0.017*
No	27 (40)	23 (50)	4 (18)	
Splenomegaly BLCM				
≥5 cm	22 (32)	10 (44)	12 (67)	*0.036*
<5 cm	19 (28)	13 (56)	6 (33)	

Abbreviation: BM, bone marrow; BLCM, below left costal margin; Hb, hemoglobin; IQR, interquartile range; PB, peripheral blood; PMF, primary myelofibrosis; LDH, lactate dehydrogenase; ns, not significant; SMF, secondary myelofibrosis; WBC, White Blood Cell Count.

Table 6. Prognostic risk assessment

	Overall (n=68)	PMF (n=46)	SMF (n=22)
IPSS			
Low		32 (70)	-
Intermediate-1		9 (20)	
Intermediate-2		3(6)	
High		2 (4)	
DIPSS			
Low		33 (72)	-
Intermediate-1		10 (22)	
Intermediate-2		3 (6)	
High		0	
MYSEC-PM			
Low		-	7 (32)
Intermediate-1			13 (59)
Intermediate-2			2 (9)
High			
MIPSS70			
Low	18 (25)	17 (37)	1 (5)
Intermediate	45 (68)	25 (54)	20 (91)
High	5 (7)	4 (7)	1 (5)

Abbreviations: IPSS, International Prognostic Scoring System; DIPSS, Dynamic International Prognostic Scoring System; MYSEC-PM, Myelofibrosis Secondary to PV and ET–Prognostic Model; MIPSS70, Mutation-enhanced international prognostic score system.

Table 7. Type of driver mutations and number of non-driver mutations according to the type of MF

	Overall	PMF	SMF	
	No. of patients=68	No. of patients=46	No. of patients=22	p value
Driver Mutations, n (%)				
JAK2 V617F	35 (52)	22 (48)	13 (59)	ns
CALR	19 (28)	13 (28)	6 (27)	
MPL	3 (4.4)	1 (2)	2 (9)	
Triple negative	10 (15)	10 (22)	0	
MPL+JAK2 V617F	1 (1.5)	0	1 (5)	
VAF (%) of driver mutations (median; IQR; min-max)				
JAK2 V617F	28.4 (14.9-49.3; 1.4-87.8)	21.4 (9.4-39.4;1.4-81.6)	43 (39.5-41.7; 38.6-41.8)	*0.023*
CALR	38.1 (29.8-41.5; 10.9-47.2)	34.4 (25.8-41.1; 10.9-47.2)	40.5 (39.5-41.7;38.6-41.8)	ns
MPL	42 .1 (29.8-68.7;27.2-75.6)	27.2 (nc)	47.8 (36.3-nc; 36.3-75.6)	ns
Number of non-driver mutations (median, IQR; min-max)	1 (0-2; 0-6)	1 (0-2;0-6)	1 (0-2;0-4)	ns

Abbreviation: IQR, interquartile range; max, maximum; MF, myelofibrosis; min, minimum; ns, not significant; PMF, primary myelofibrosis; SMF, secondary myelofibrosis; VAF, variant allele frequencies.

Table 8. Frequencies of variants detected in MF patients according to their functional group

Biologic category	Gene	All patients (n=68)	PMF (n=46)	SMF (n=22)	p value
Signal transduction	CBL	4 (5.8%)	2	2	ns
	JAK2	1 (1.5%)	1	0	ns
	CSF3R	3 (4.4%)	2	1	ns
	HRAS	1 (1.5%)	1	0	-
	FLT3	1 (1.5%)	1	0	-
	NRAS	2 (2.9%)	0	2	-
RNA splicing	SF3B1	2 (2.9%)	1	1	ns
	SRSF2	2 (2.9%)	2	0	-
	U2AF1	1 (1.5%)	0	1	-
	ZRSR2	1 (1.5%)	1	0	-
DNA methylation	DNMT3A	7 (10.3%)	5	2	ns
	TET2	14 (20.6%)	7	7	ns
Histone modification	ASXL1	11 (16%)	7	4	ns
	EZH2	1 (1.5%)	0	1	-
Transcription Regulation	RUNX1	2 (2.9%)	1	1	ns
	SETBP1	2 (2.9%)	1	1	ns
	TP53	3 (4.4%)	2	1	ns
	WT1	1 (1.5%)	1	0	-
	ETV6	2 (2.9%)	2	0	-
	CEBPA	4 (5.8%)	4	0	-

Abbreviation: ns, not significant; PMF, primary myelofibrosis; SMF, secondary myelofibrosis

Table 9. Significant different variables according to *ASXL1* mutational status

Variable	*ASXL1* mut No. of patients=11	*ASXL1* wt No. of patients=57	*p value*
Monocytes x 10^9/L			
Median (IQR)	0.6 (0.5-0.7)	0.3 (0.2-0.5)	*<0.001*
Lymphocytes x 10^9/L			
Median (IQR)	2.1 (1.6-2.6)	1.5 (1.1-1.9)	*0.015*
LDH (U/L)			
Median (IQR)	450 (350-703)	260 (220-320)	*<0.001*
Spleen LD by ultrasound (cm)			
Median (IQR)	19 (17-22)	15 (13-18)	*0.035*

Abbreviation: IQR, interquartile range; LD, longitudinal diameter; mut, mutated; wt, wild type

Table 10. Significant different variables according to *DNMT3A* mutational status

Variable	*DNMT3A* mut No. of patients=7	*DNMT3A* wt No. of patients=61	*p value*
WBC x 10^9/L			
Median, (IQR)	10.9 (9-14.2)	7.2 (6.1-9.5)	*0.043*
Neutrophils x 10^9/L			
Median, (IQR)	7.3 (7-10.5)	5.0 (3.9-6.6)	*0.047*
Platelets x 10^9/L			
Median (IQR)	1144 (439-1375)	260 (220-320)	*0.032*

Abbreviations: IQR, interquartile range; mut, mutated; wt, wild type

Table 11. Characteristics of ruxolitinib-treated patients

Variable	No. of patients =21
Gender, n (%)	
Male,	11 (57)
Female	10 (43)
Type of MF, n (%)	
PMF	9
SMF	12
Age at diagnosis, years	
Median, (IQR)	57 (44-59)
Hb, g/Dl	
Median, (IQR)	11.3 (10.6-13.6)
WBC × 10^9/L	
Median, (IQR)	8.2 (6.1-14.3)
Neutrophils x 10^9/L	
Median, (IQR)	6.1 (4.2-10.4)
Monocytes x 10^9/L	
Median, (IQR)	0.4 (0.3-0.6)
Lymphocytes x 10^9/L	
Median, (IQR)	1.4 (1.9-1.0)
Platelet count × 10^9/L	
Median, (IQR)	376 (237-487)
LDH (U/L)	
Median, (IQR)	350 (297-558)
Driver mutation, n (%)	
JAK2 V617F	12 (57)
CALR	5 (24)
MPL	1 (5)
TN	3 (14)

HMR mutations, n (%)	
≥1	8 (38%)
≥2	2 (10%)
BM fibrosis grade, n (%)	
Grade 1	5 (24)
Grade 2	4 (19)
Grade 3	12 (57)
Starting dose of ruxolitinib	
20 mg BID	16 (77)
15 mg BID	2 (9.5)
10 mg BID	2 (9.5)
5 mg BID	1 (4.7)

Abbreviation: BID, bis in die; BM, bone marrow; Hb, hemoglobin; HMR, high molecular risk; IQR, interquartile range; PMF, primary myelofibrosis; LDH, lactate dehydrogenase; ns, not significant; SMF, secondary myelofibrosis; WBC, White Blood Cell Count.

Figures

Figure 1. Gene panel

ABL1 (4-9)	ASXL1 (10,12,13)	BRAF (15)	CALR (9)	CBL (8,9)
CEBPA (all)	CSF3R (all)	DNMT3A (all)	ETV6 (all)	EZH2 (all)
FLT3 (13-15,20)	HRAS (2,3)	IDH1 (4)	IDH2 (4)	JAK2 (all)
KIT (2,8-11,13,17,18)	KRAS (2,3)	MPL (10)	NPM1 (10,11)	NRAS (2,3)
PTPN11 (3,7-13)	RUNX1 (all)	SETBP1 (4)	SF3B1 (10-16)	TET2 (all)
SRSF2 (1)	TET2 (all)	U2AF1 (2,6)	WT1 (6-10)	ZRSR2 (all)

Figure 2. Non-driver mutations of the entire cohort
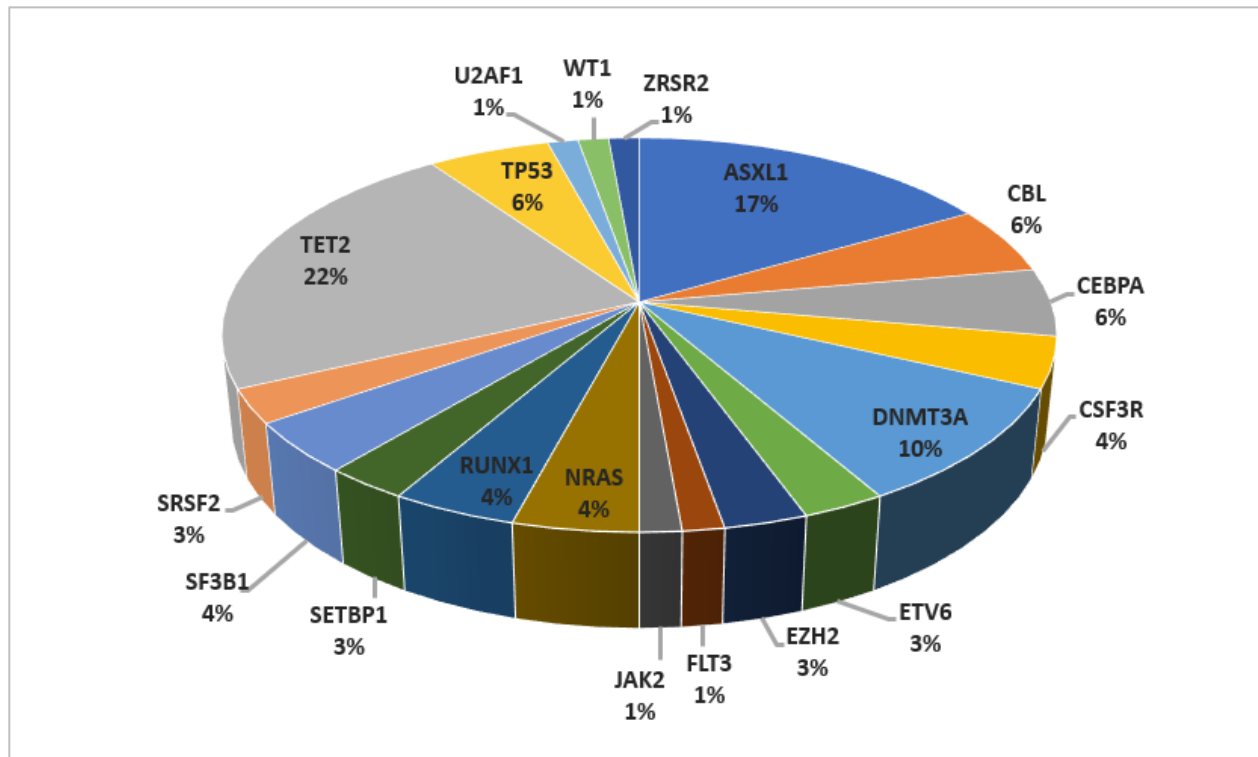
Figure 3. Functional consequences of mutations found
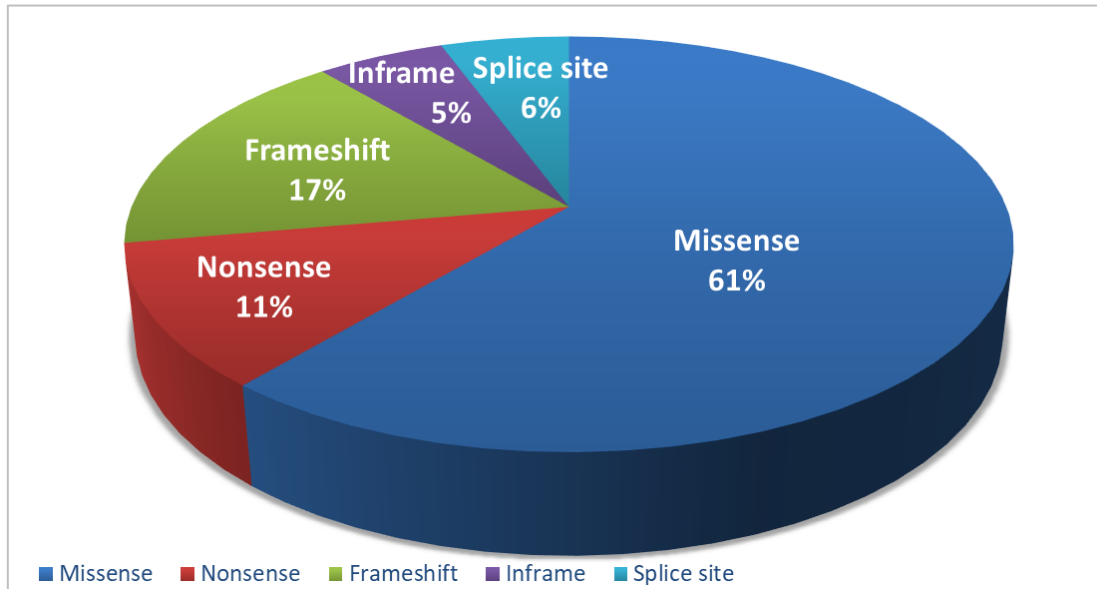
Figure 4. Proportion of patients with additional mutations according to driver mutations (JAK2 V617F versus CALR)
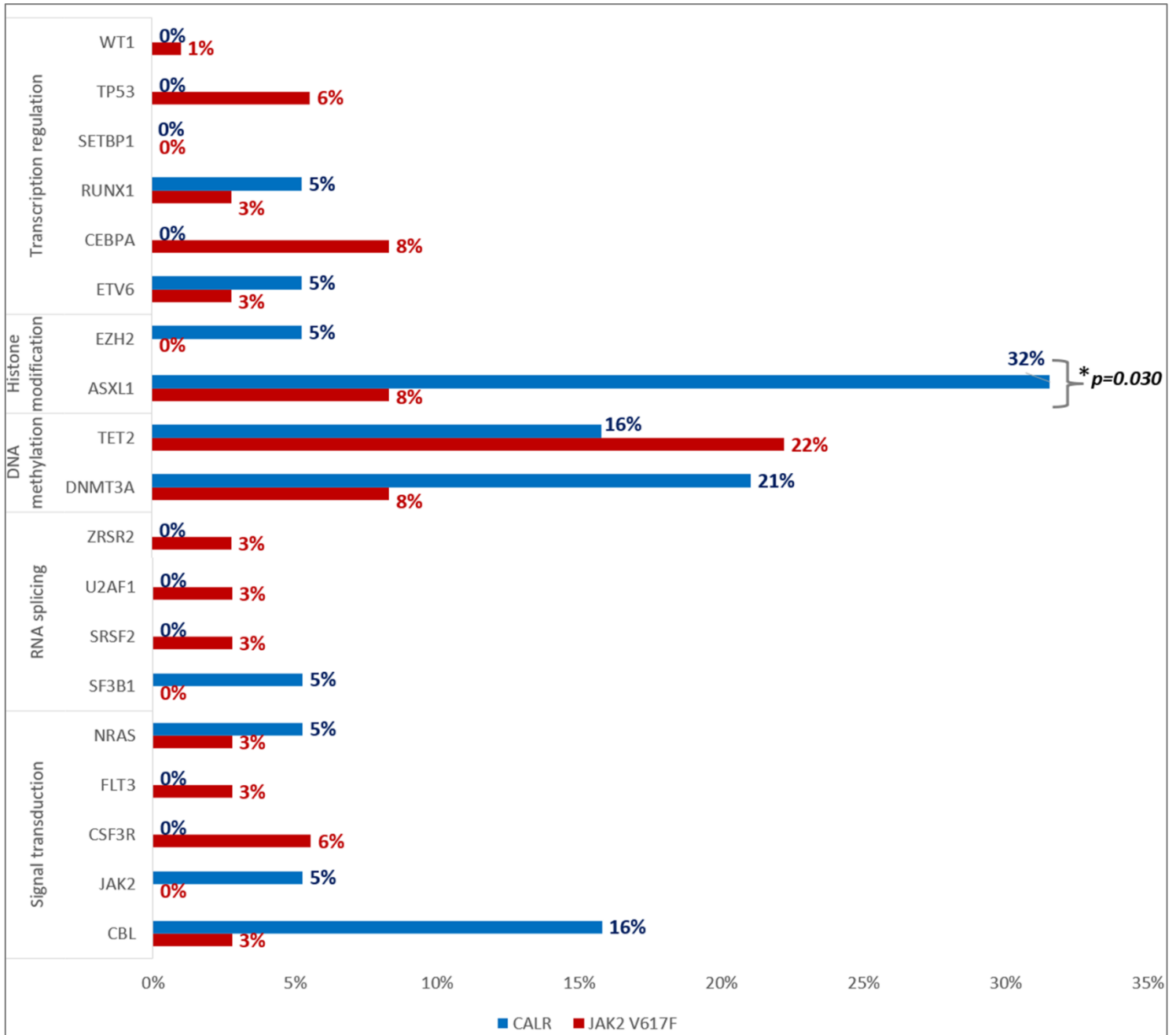
Figure 5. Risk stratification according to MIPSS70 score
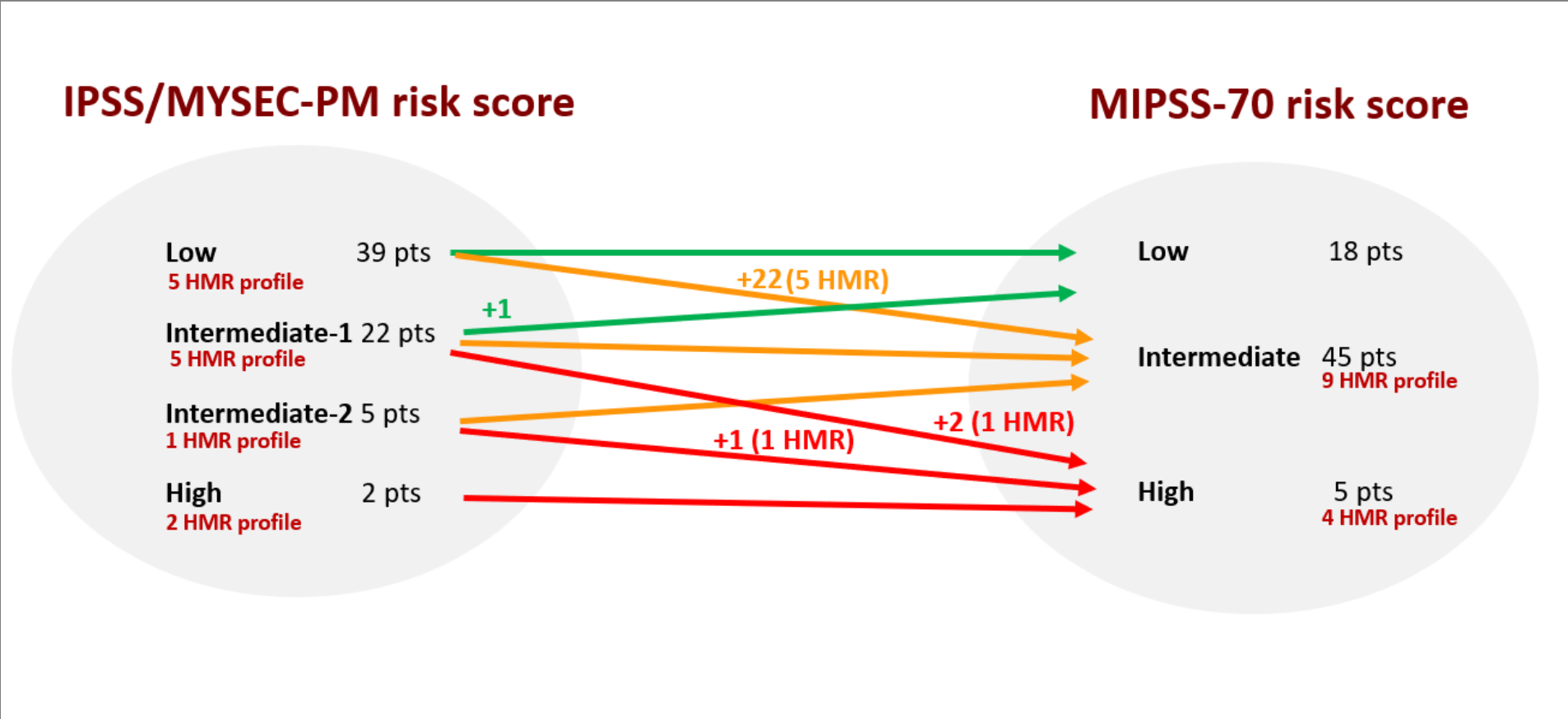
Figure 6. Graphic representation of treatment strategy according to MIPSS70 risk categories
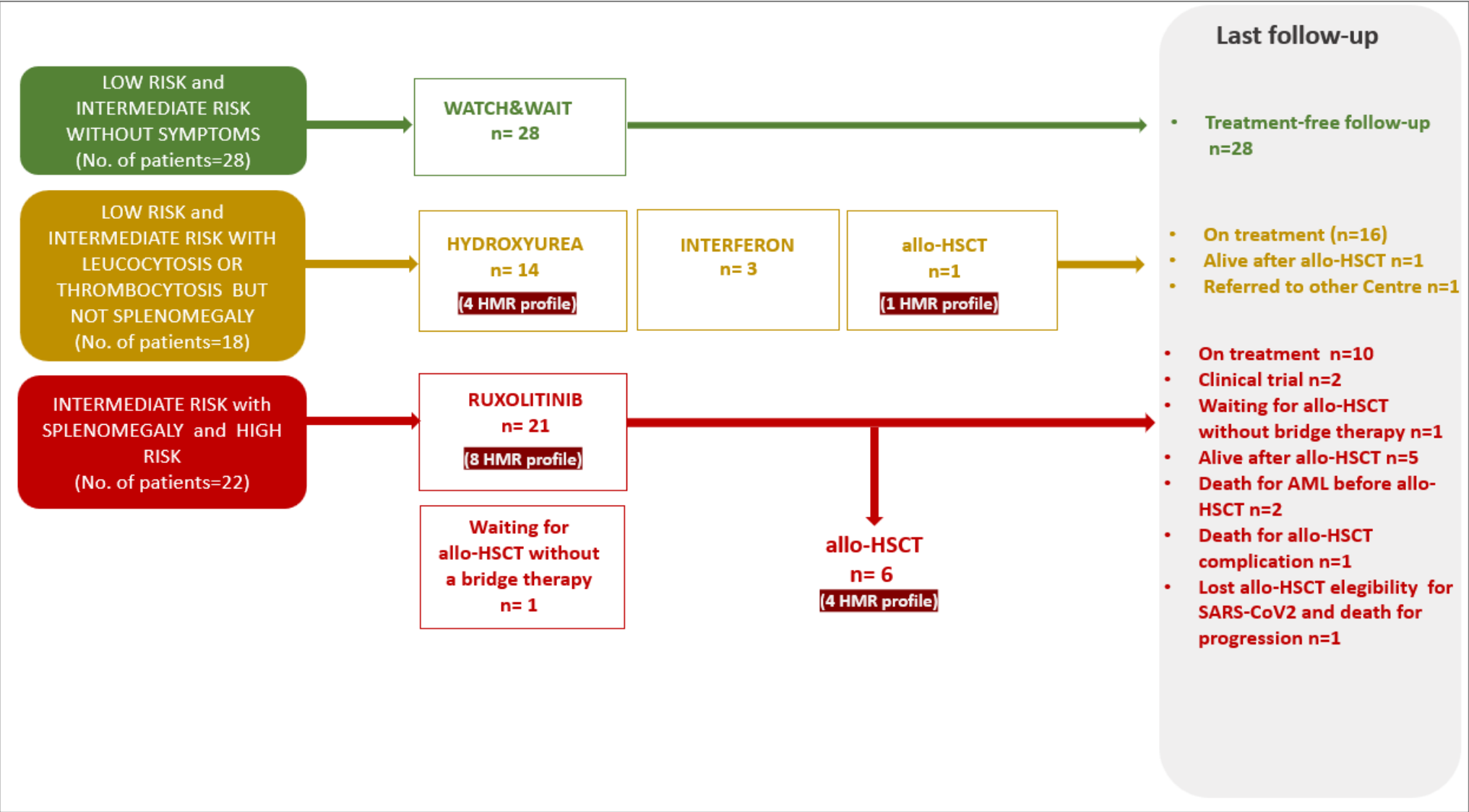
Figure 7. Time to treatment failure (TTF)

Median TTF 19.1 mo (95%CI 9.6-28.6)
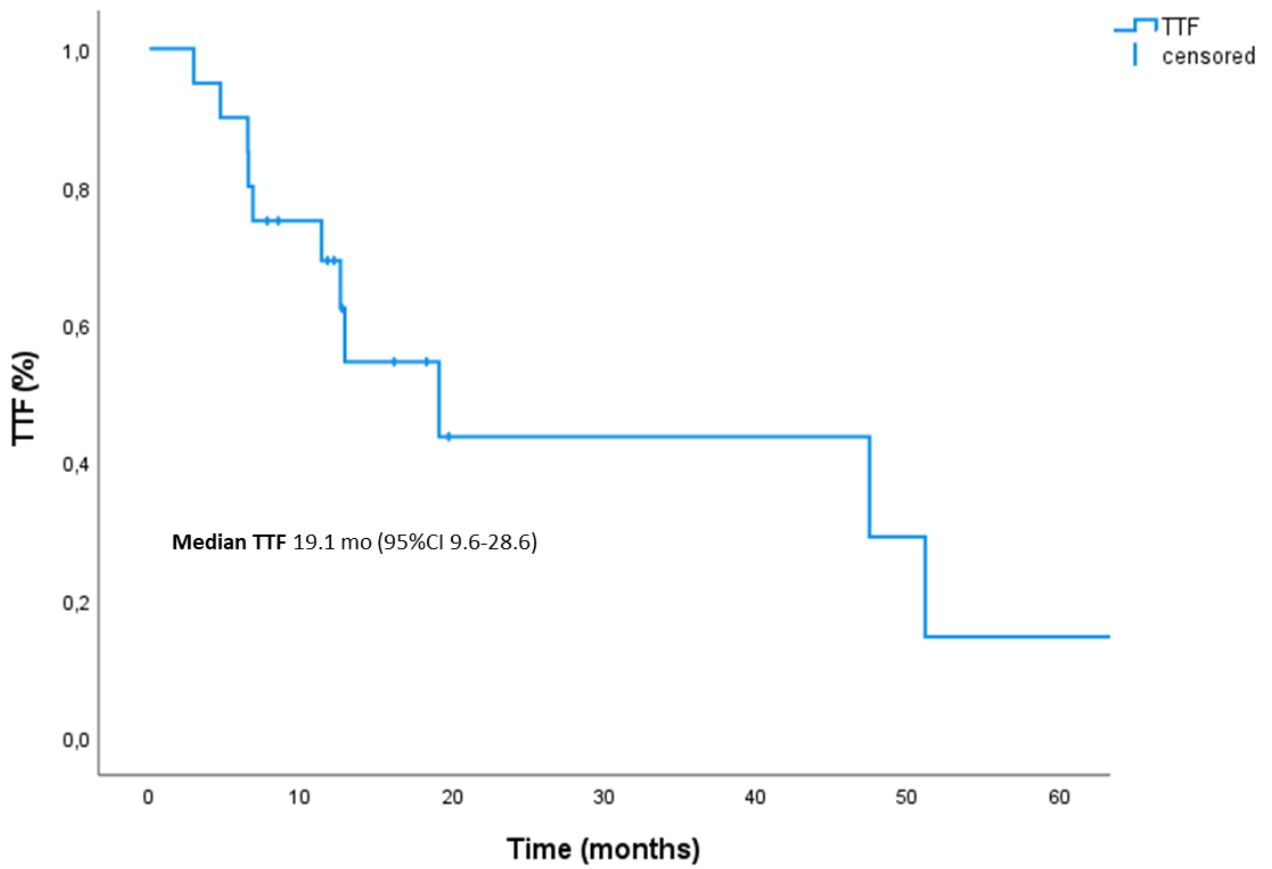
Figure 8. TTF according to MIPSS70 prognostic score

High risk Median TTF 6.5 mo (95%CI 0.8-12.1)
Intermediate risk Median TTF not reached
p=0.005
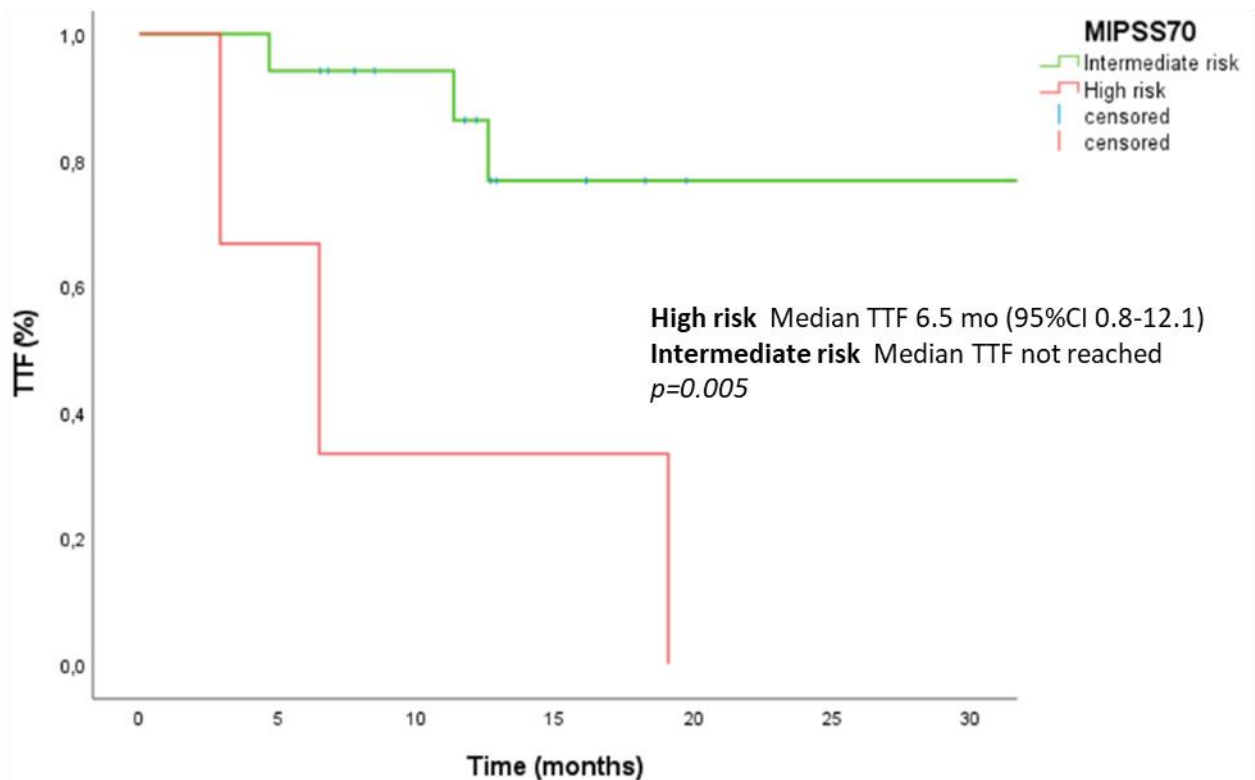
Figure 9. TTF according to the number of additional mutations
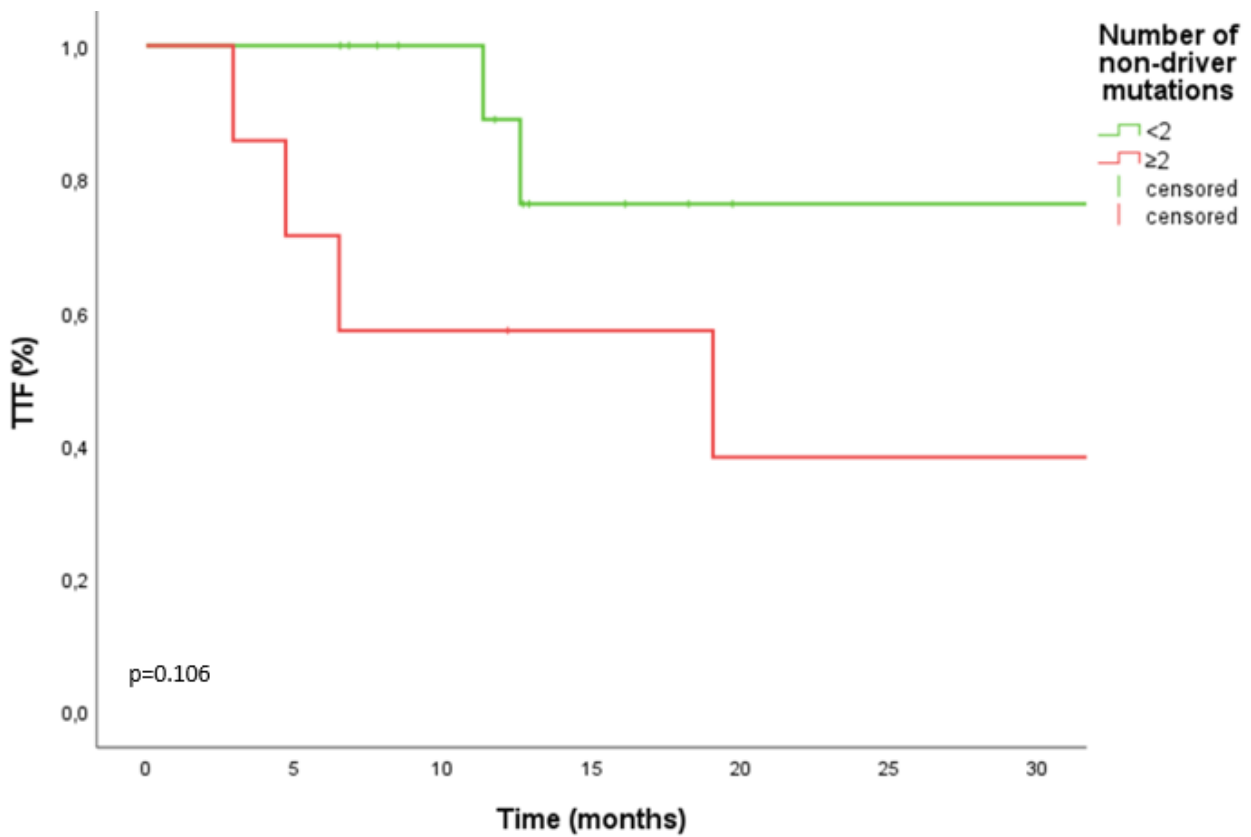
Figure 10. Overall Survival
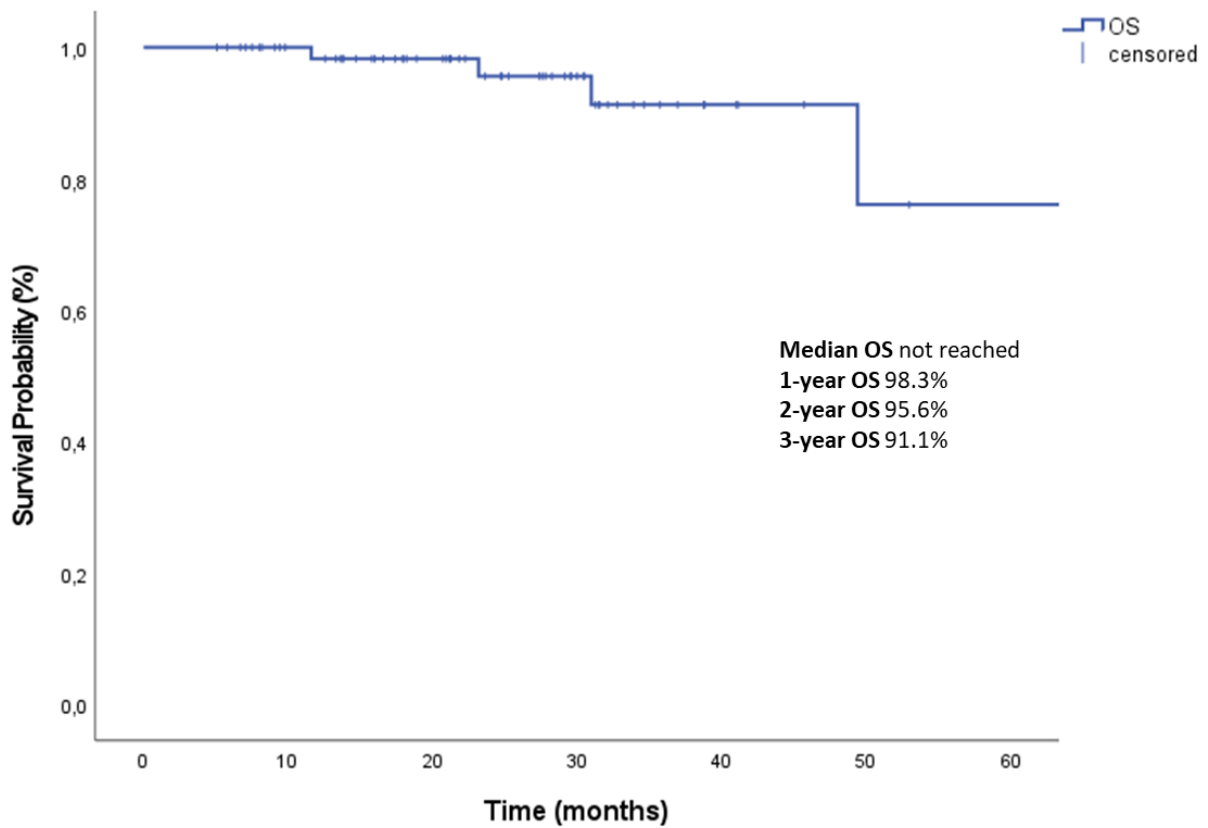
Figure 11. OS according to the age at diagnosis
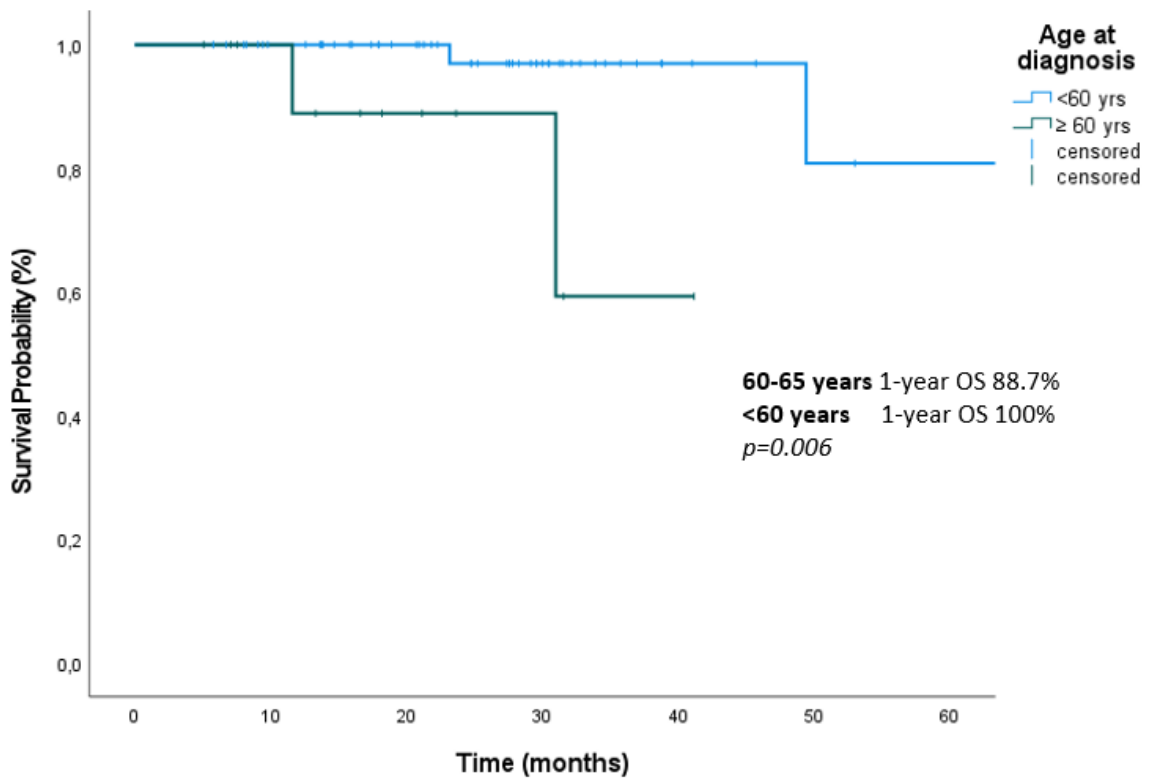
Figure 12. OS according to IPSS (A), DIPSS (B), MYSEC-PM (C) and MIPSS70 (D) prognostic risk scores

B

C

Low risk 3-year OS 100%
Intermediate risk 3-year OS 97.3%
High risk 3-year OS 50%
p=0.034

D
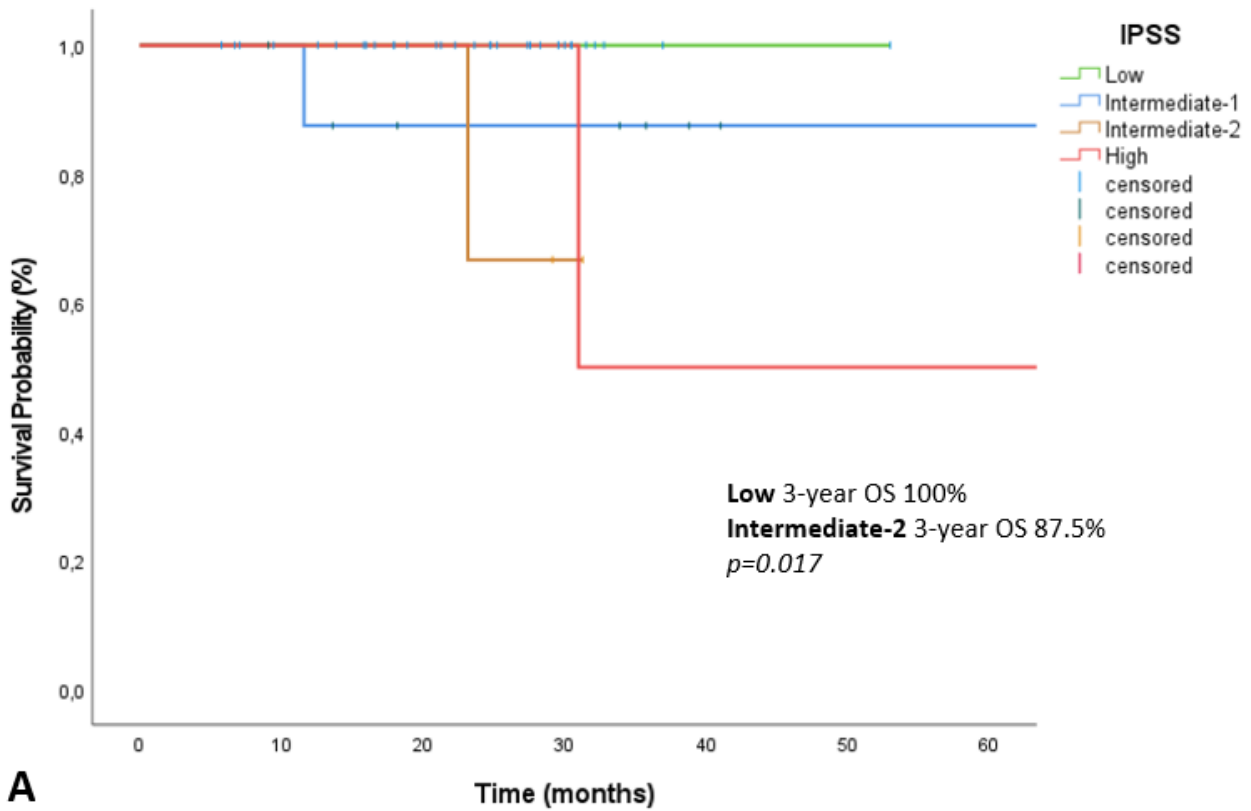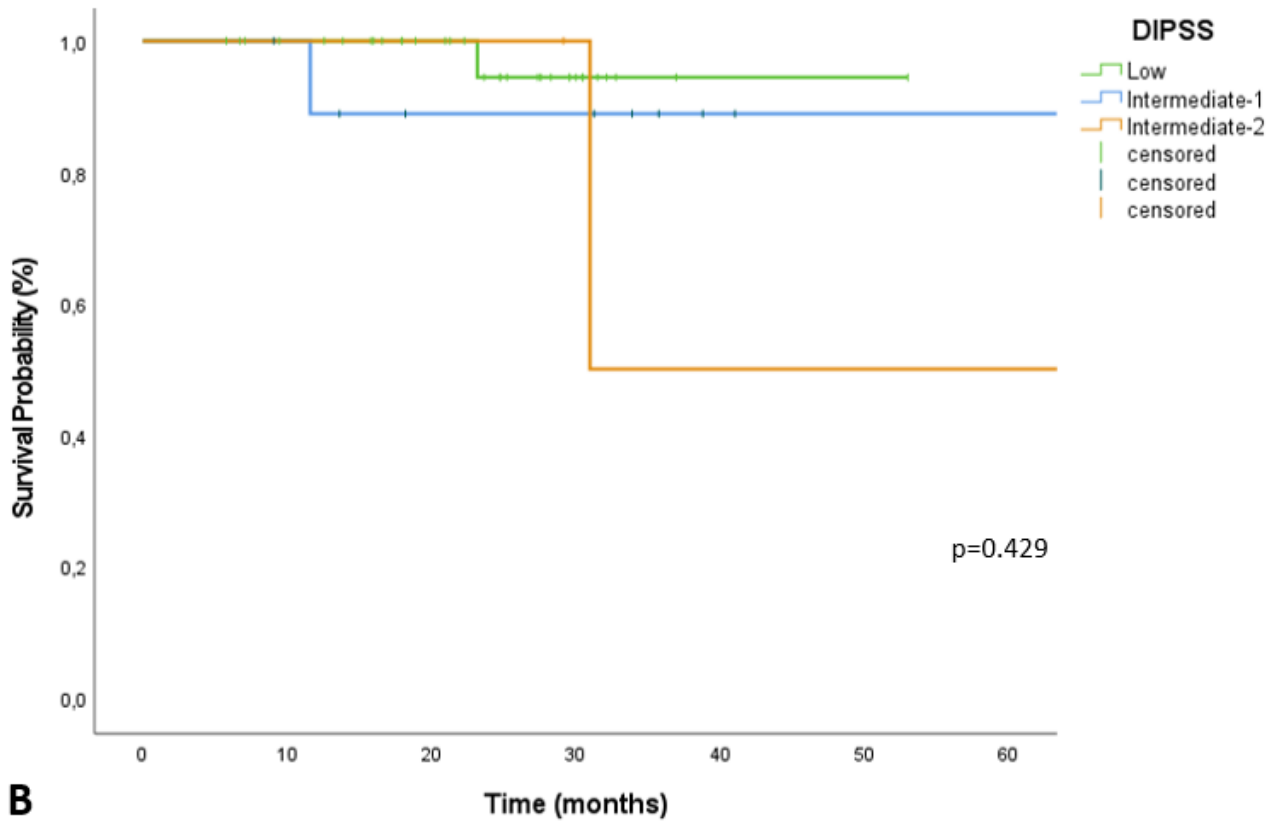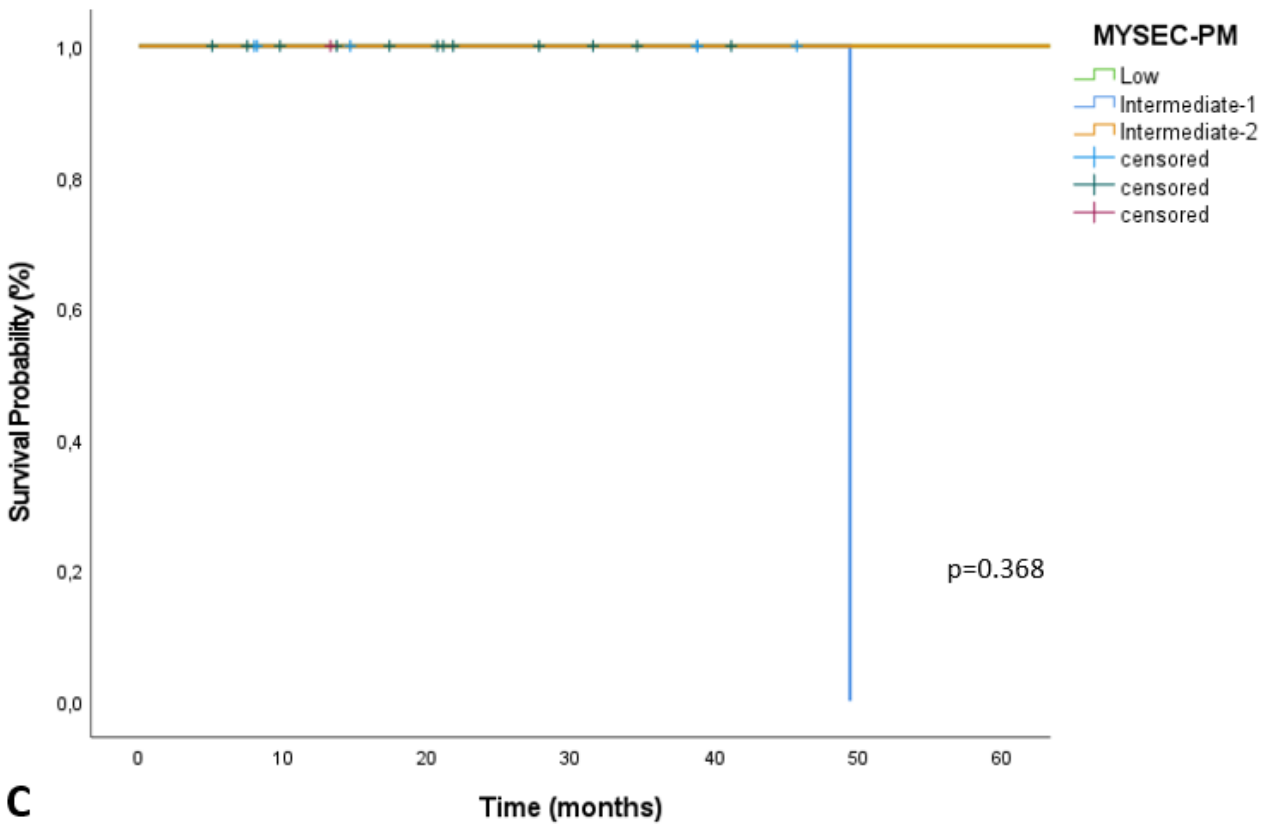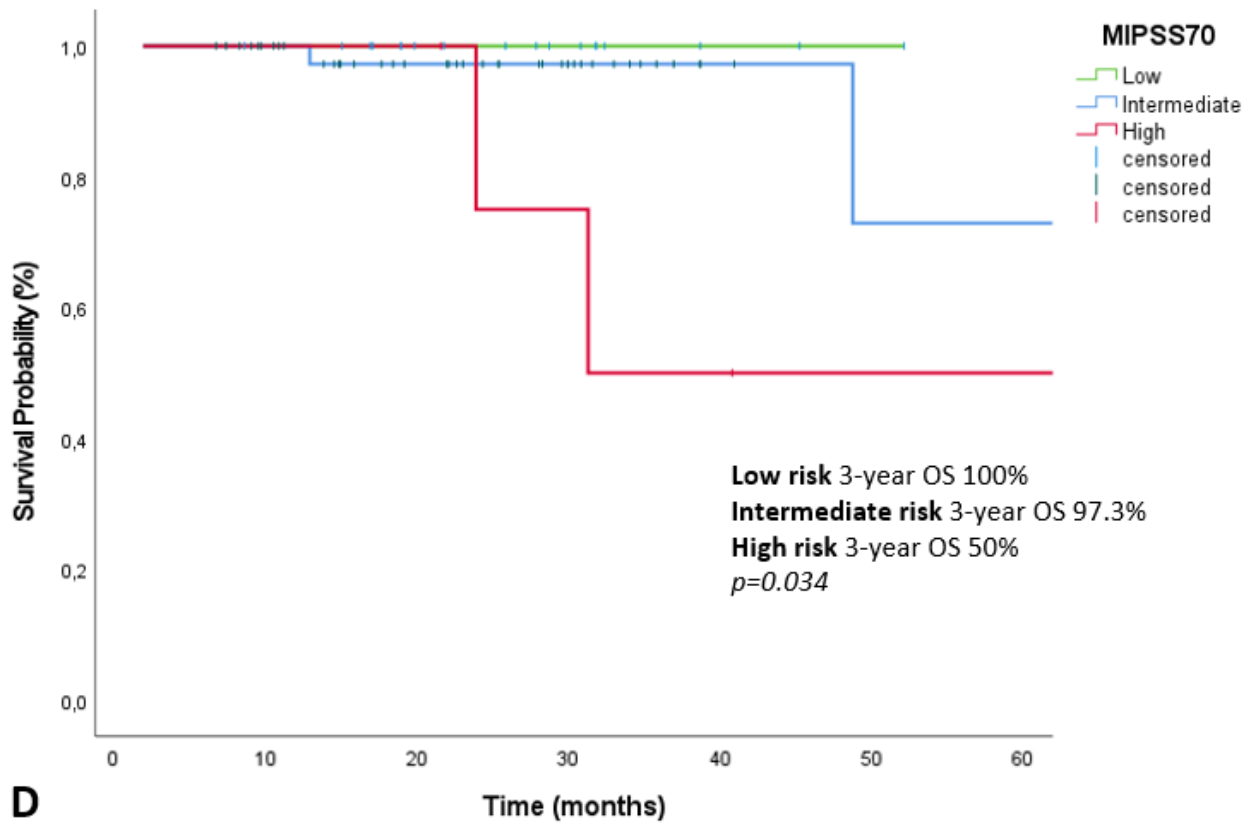
Figure 13. OS according to the presence of symptoms at diagnosis

Presence of symptoms 3-year OS 76.6%
Absence of symptoms 3-year OS 100%
p=0.021
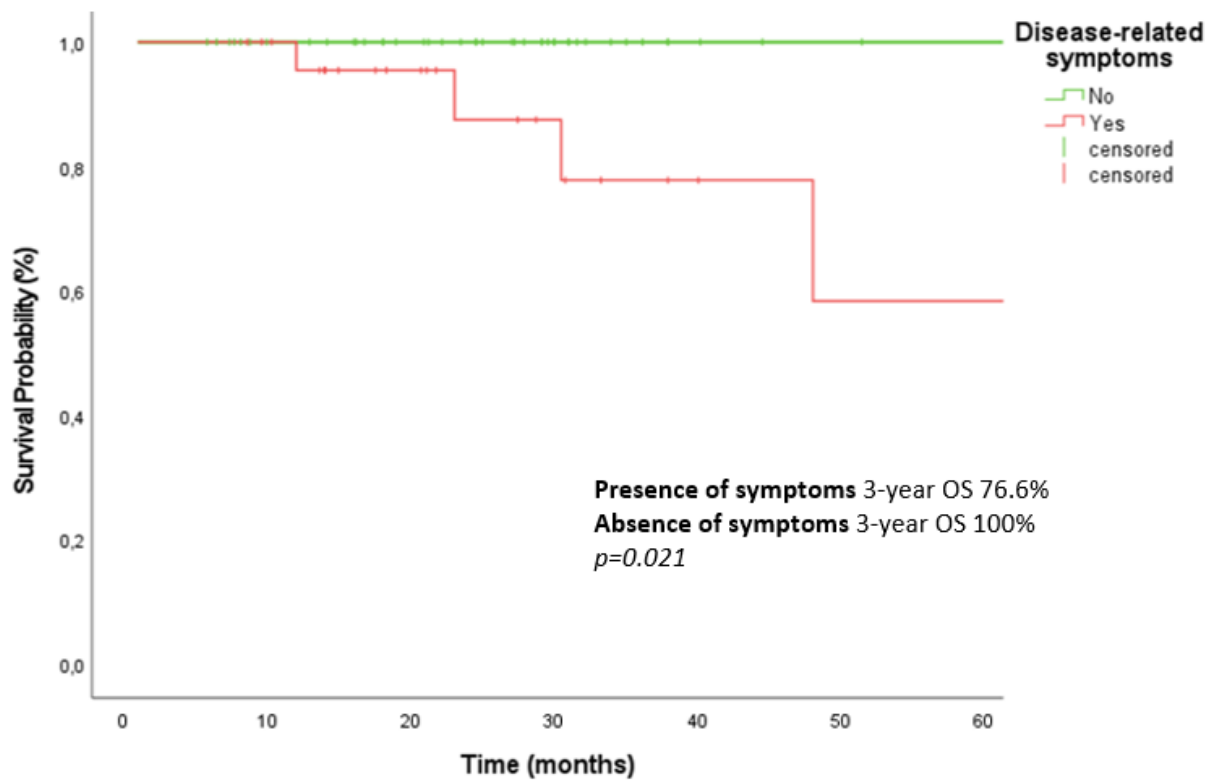
Figure 14. OS according to the type of MF
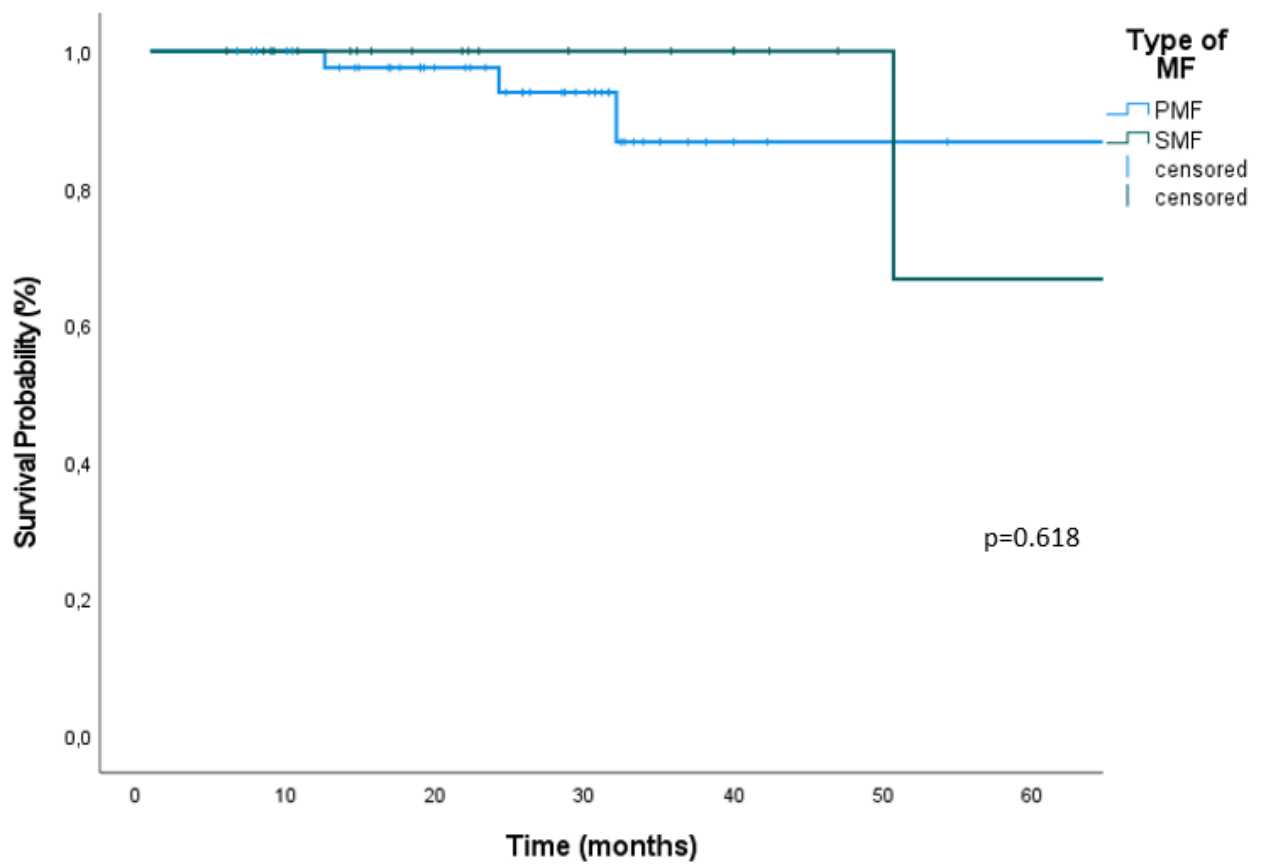
Figure 15. OS according to the type of driver mutation
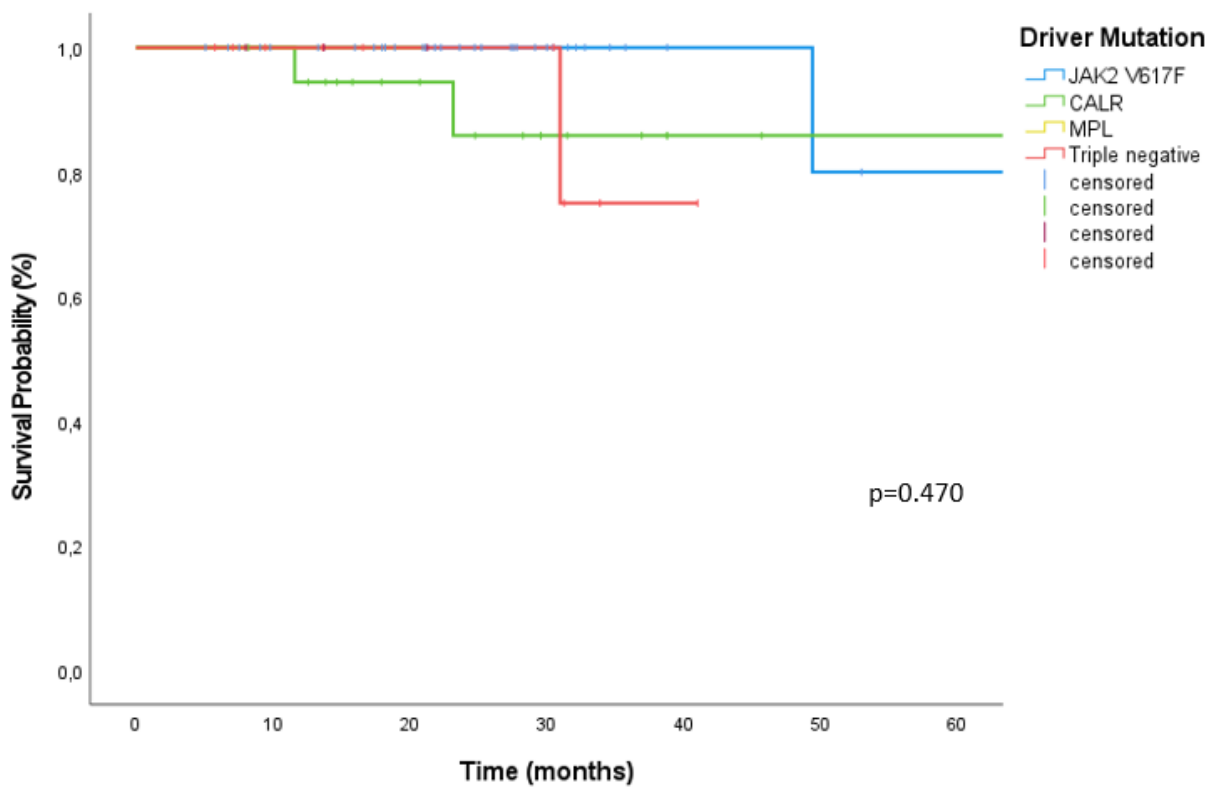
Figure 16. OS according to the presence of HMR mutations

HMR mutation Median OS 49.1 mo (95%CI 21.3-77.6)
Absence of HMR mutations Median OS not reached
p=0.032
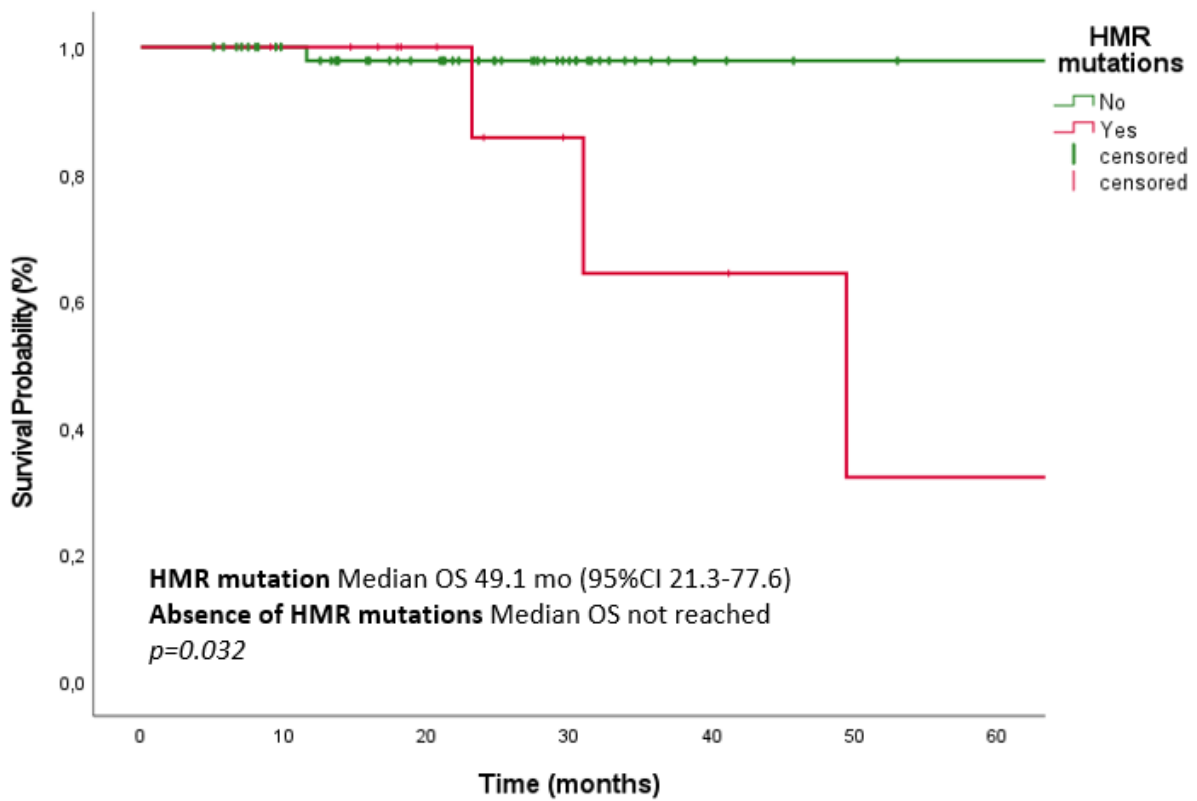
Figure 17. OS according to the number of non-driver mutations

<2 non-driver mutations 3-year OS 100%
≥2 non-driver mutations 3-year OS 78.7%
p=0.025
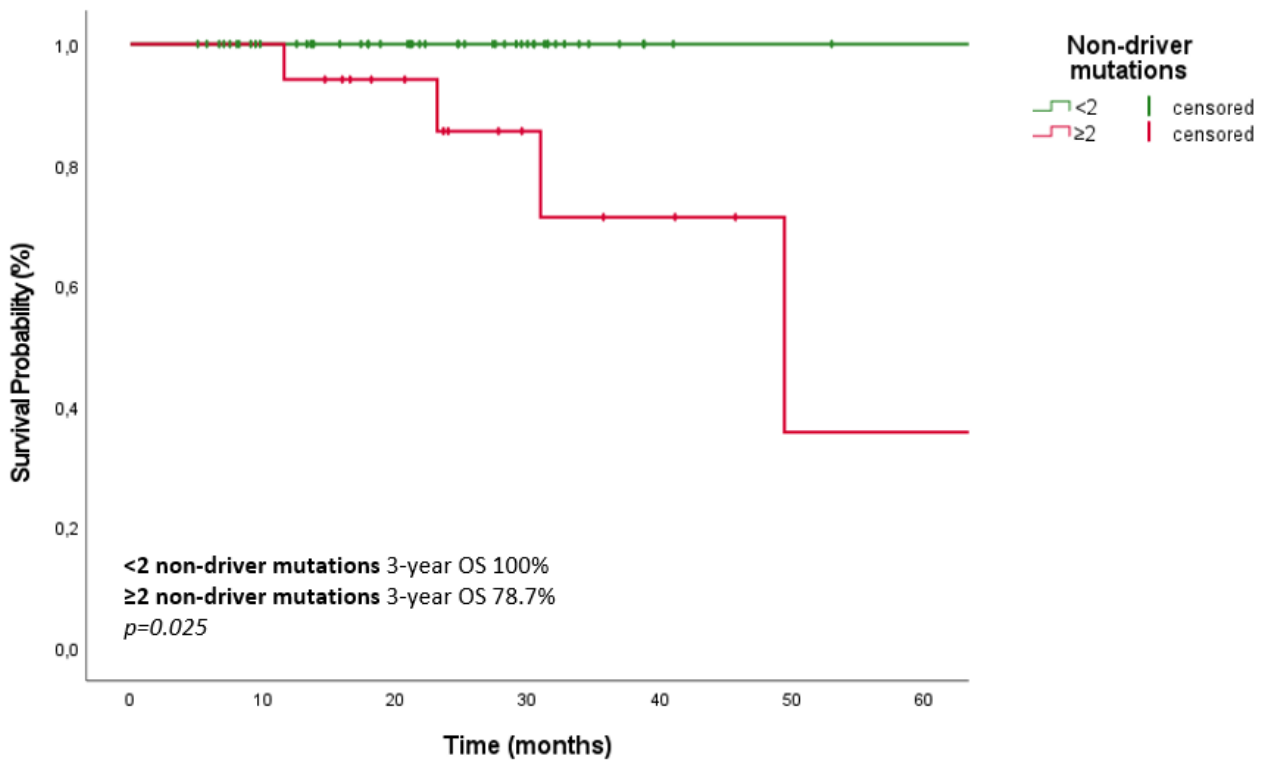
Figure 18. OS according to *ASXL1* mutation status in all patients
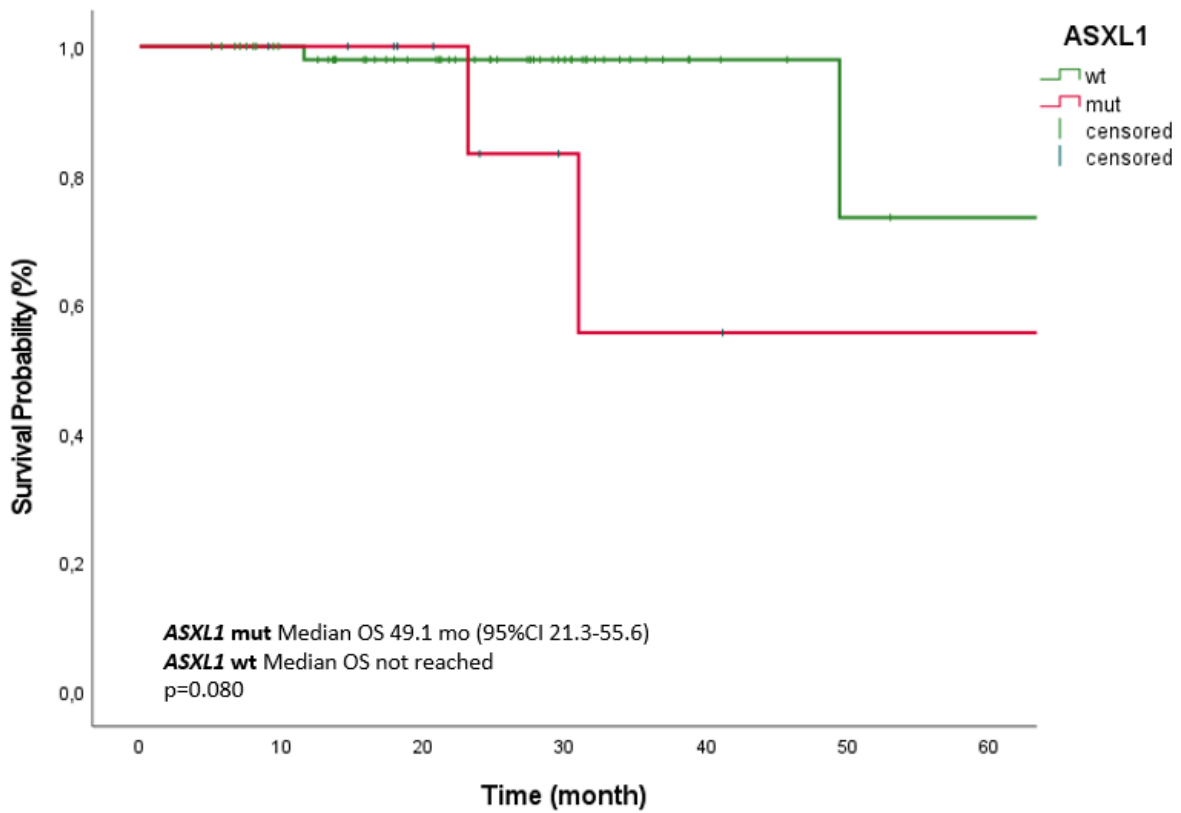
Figure 19. OS according to *RUNX1* mutation status in all patients
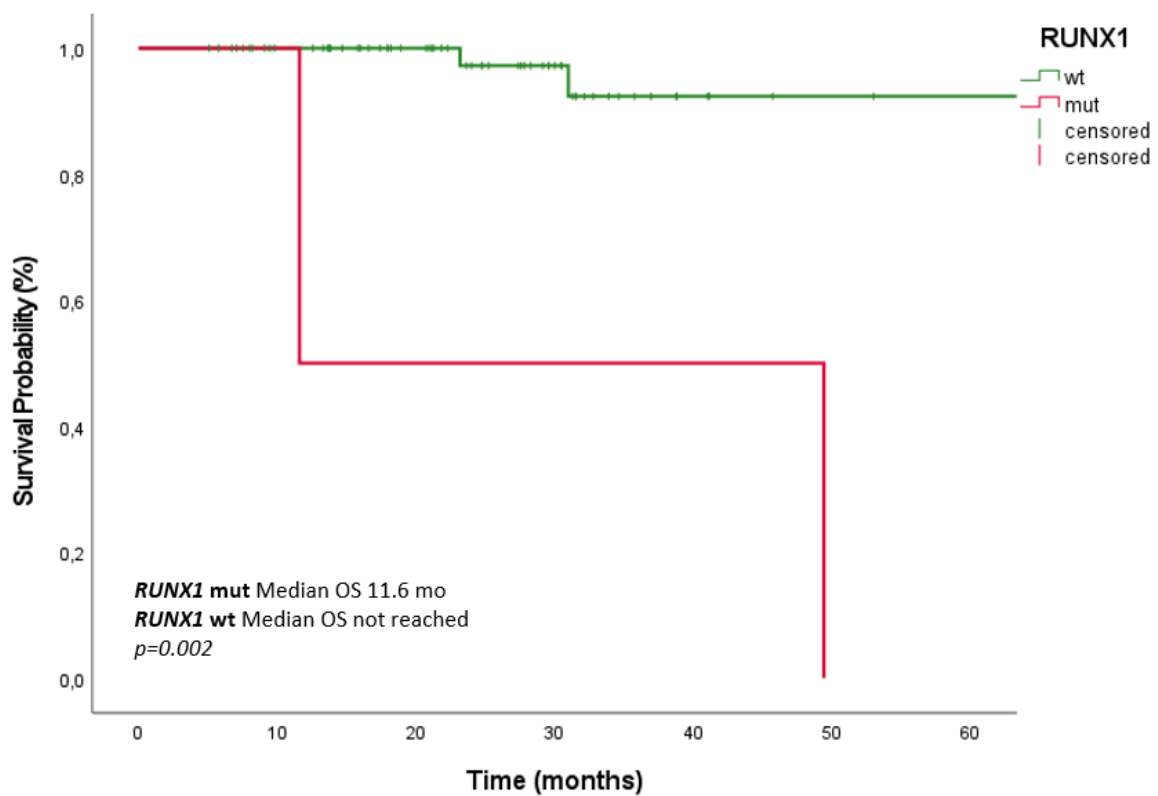
Figure 20. OS according to the presence of HMR mutations in PMF (A) and SMF (B) patients

HMR mutation Median OS 49.1 mo (95%CI 21.3-77.6)
Absence of HMR mutations Median OS not reached
p<0.001

p=0.906

A   B
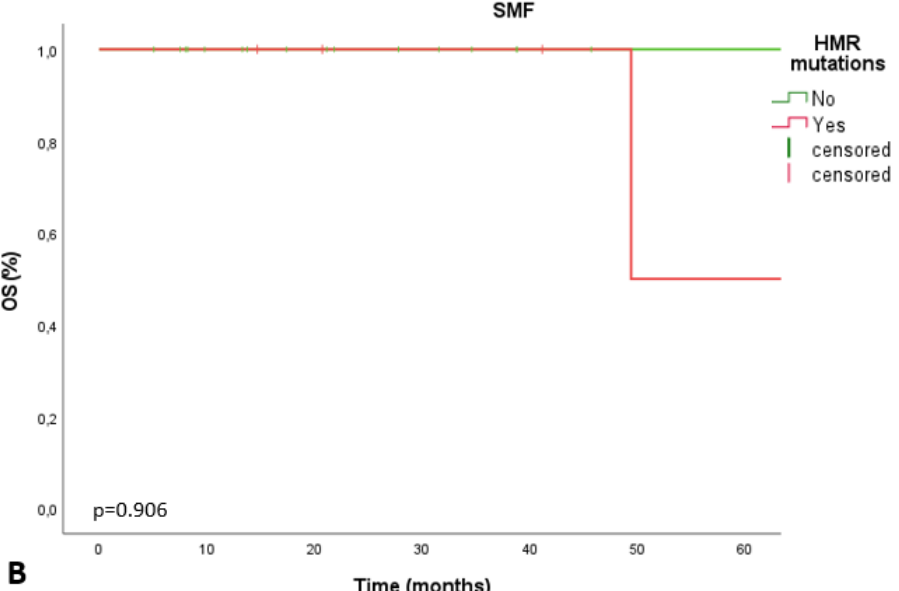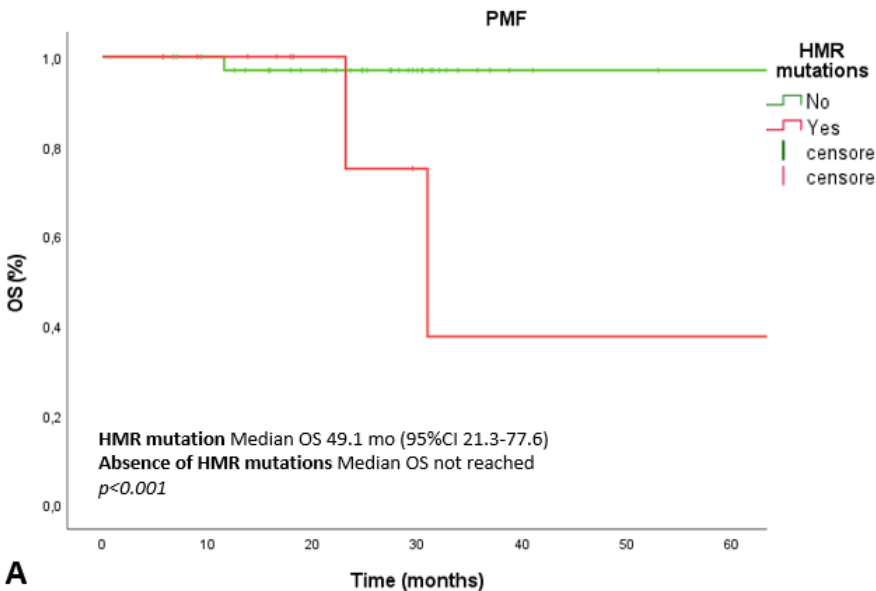
Figure 21. OS according to *ASXL1* mutation status in PMF (A) and SMF (B) patients

ASXL1 mut 2-year OS 75%
ASXL1 wt 2-year OS 97%
p=0.019

p=0.480

A   B
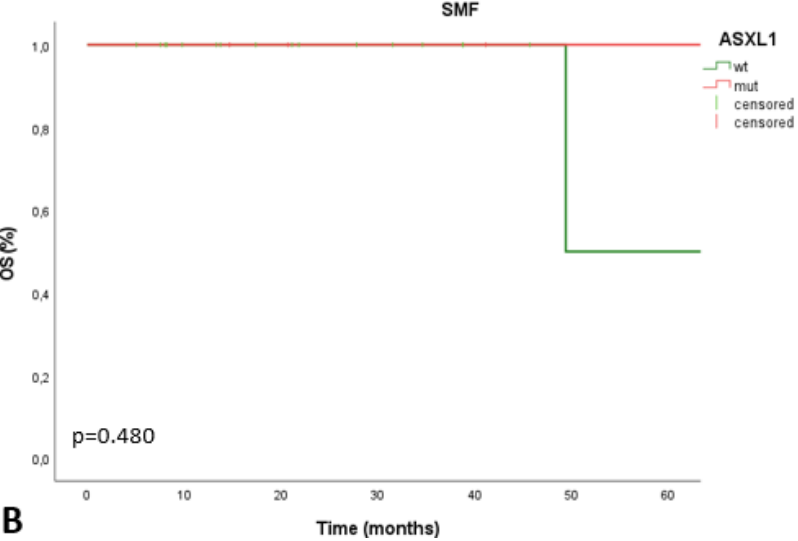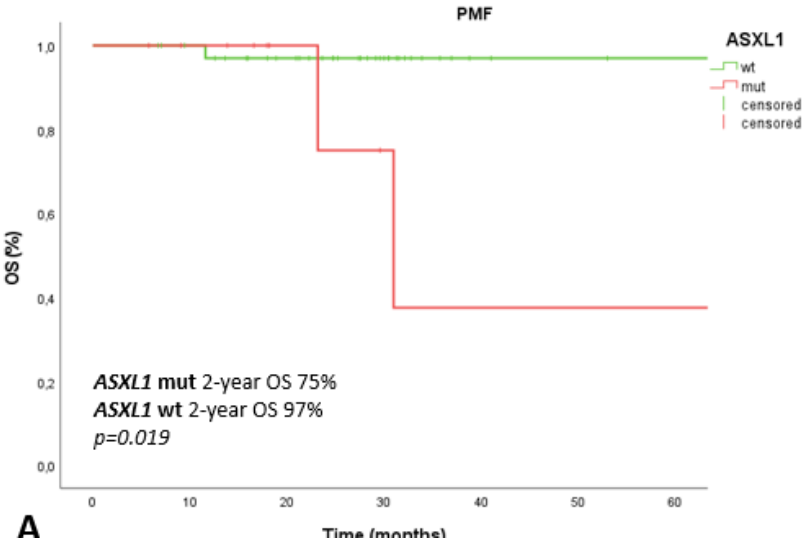
Figure 22. Event-free Survival

Median EFS not reached
6-month EFS 97%
1-year EFS 95.4%
3-year EFS 76.7%
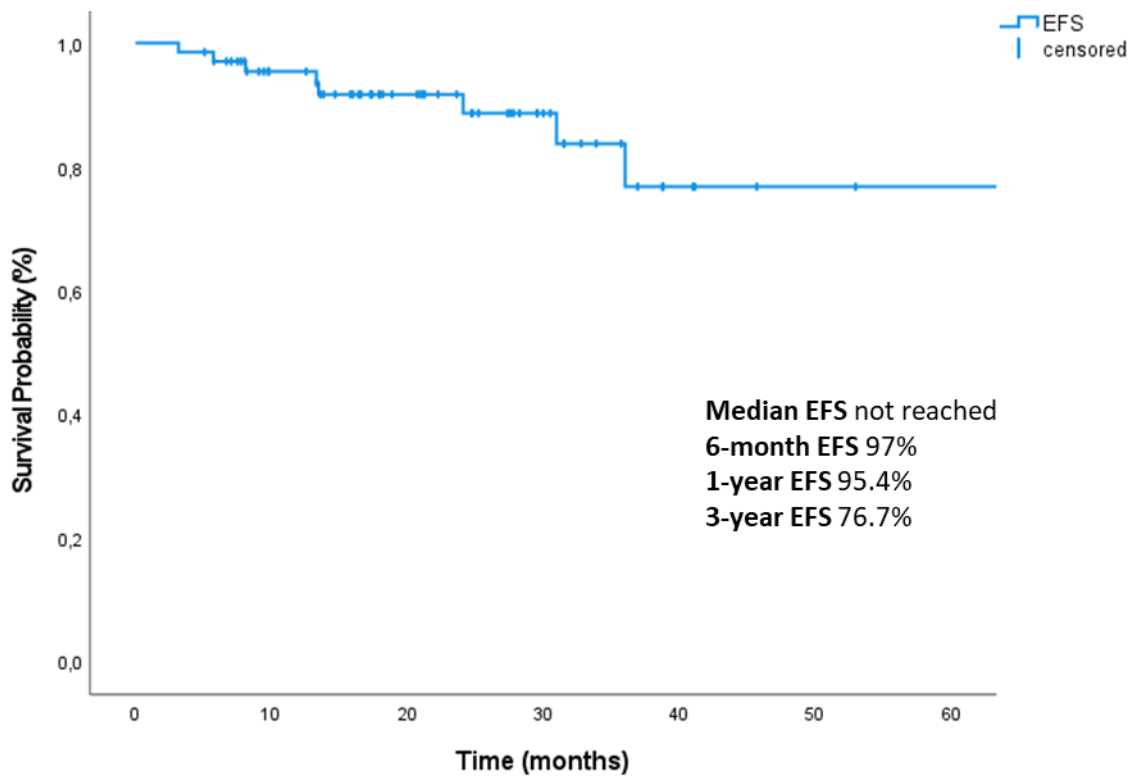
Figure 23. EFS according to IPSS prognostic score

Low risk Median EFS not reached
Int 1-2 or high risk Median EFS 24.1 mo (95%CI 2.4-88.1)
p<0.001
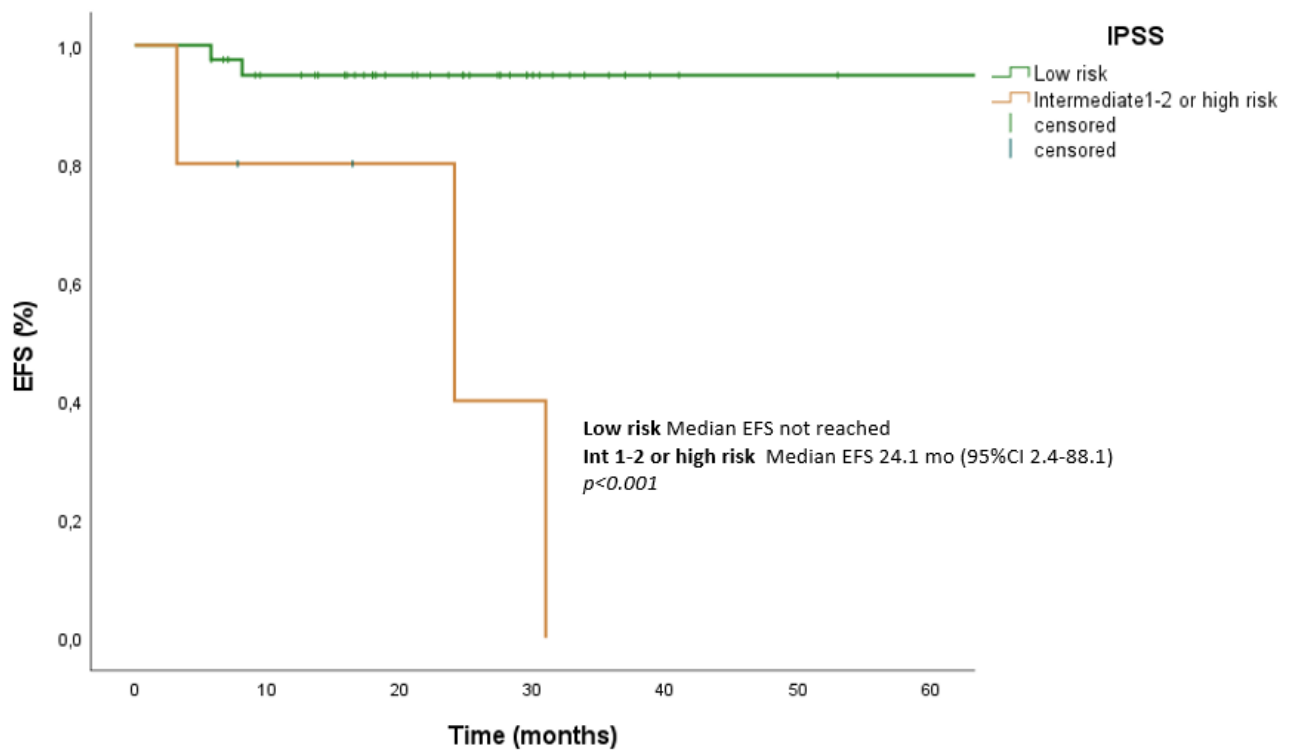
Figure 24. EFS according to MIPSS70 prognostic score
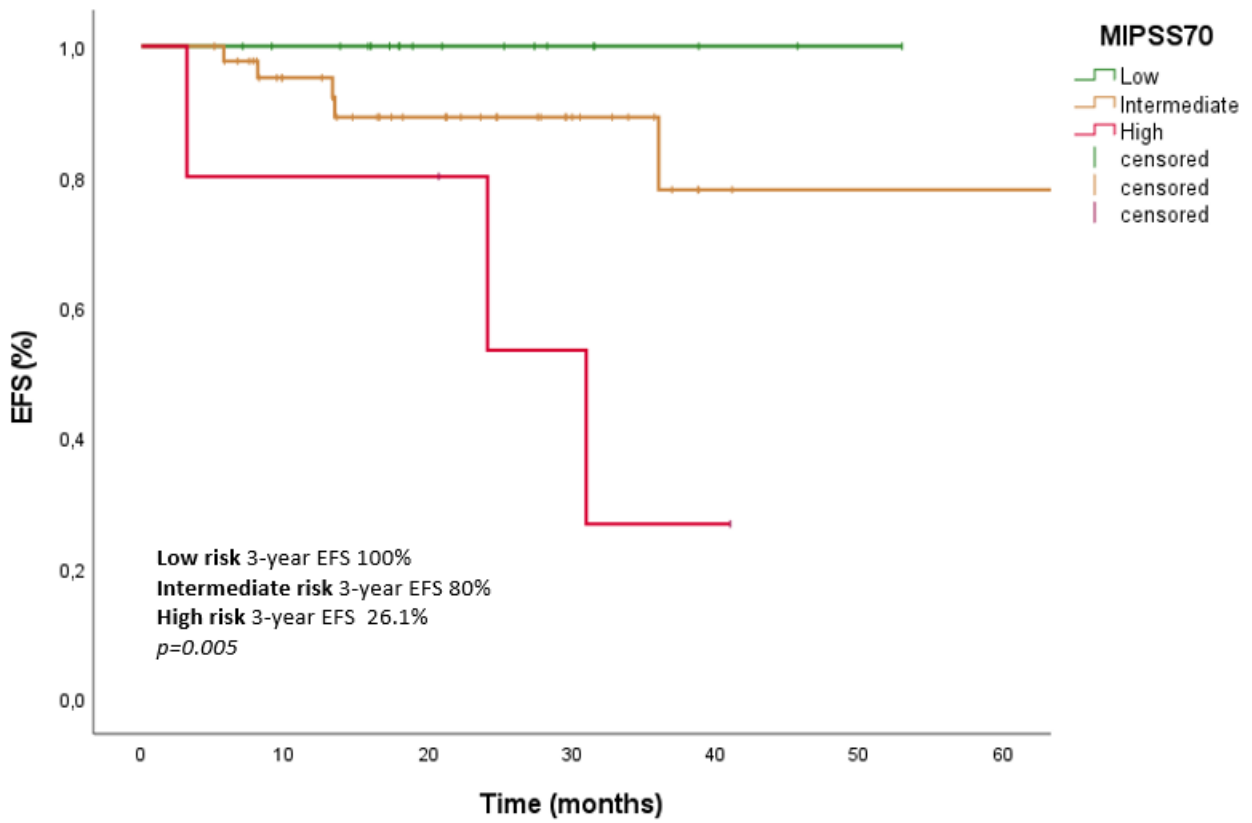
Figure 25. EFS according to the presence of disease-related symptoms
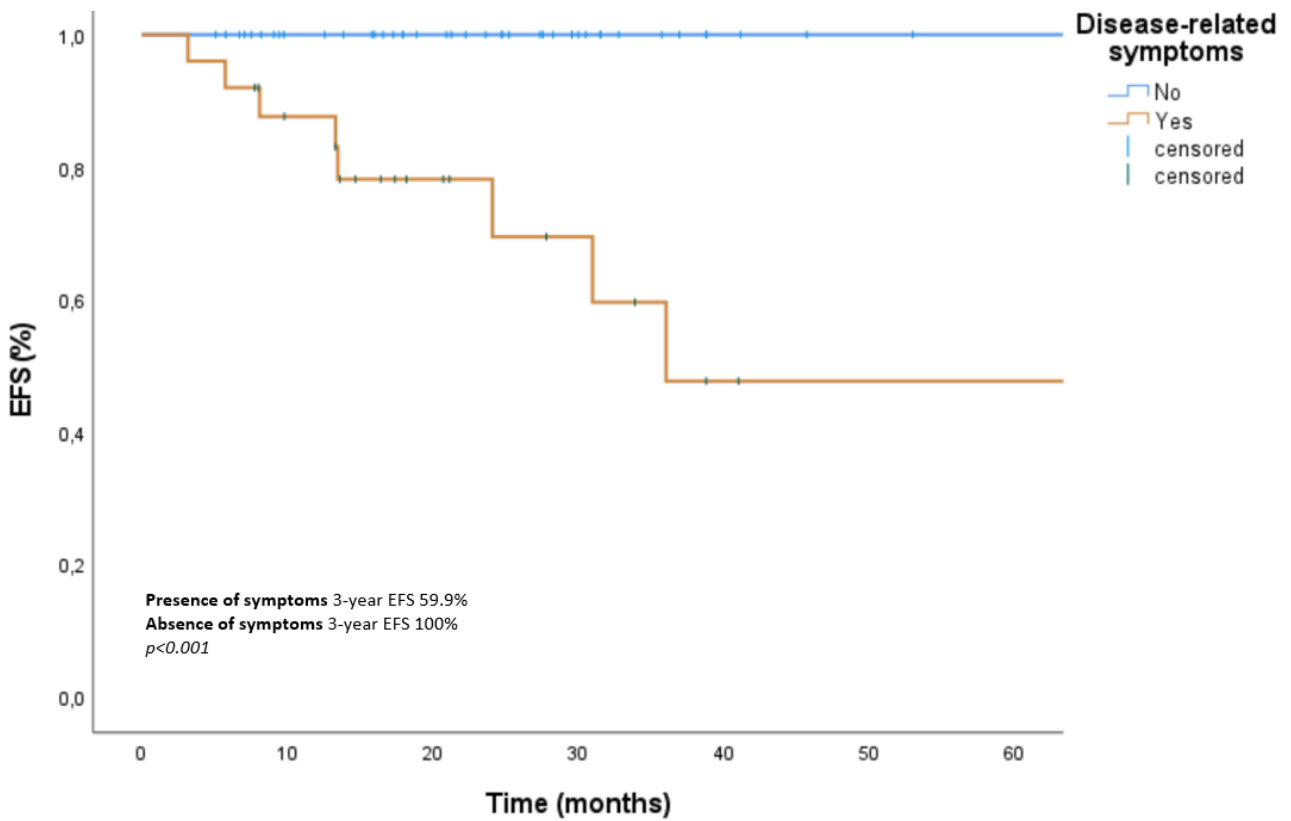
Figure 26. EFS and palpable splenomegaly
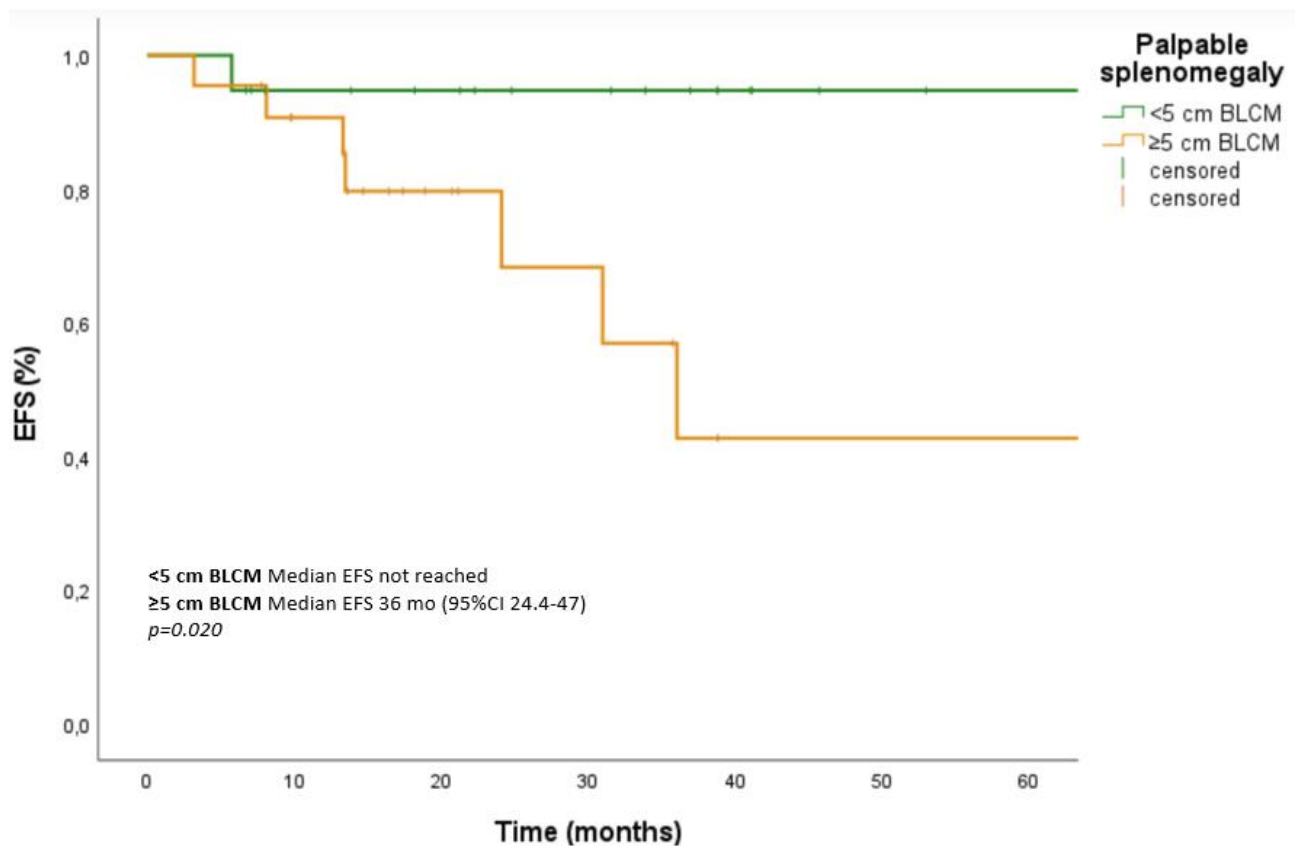
Figure 27. EFS according to the presence of HMR mutations
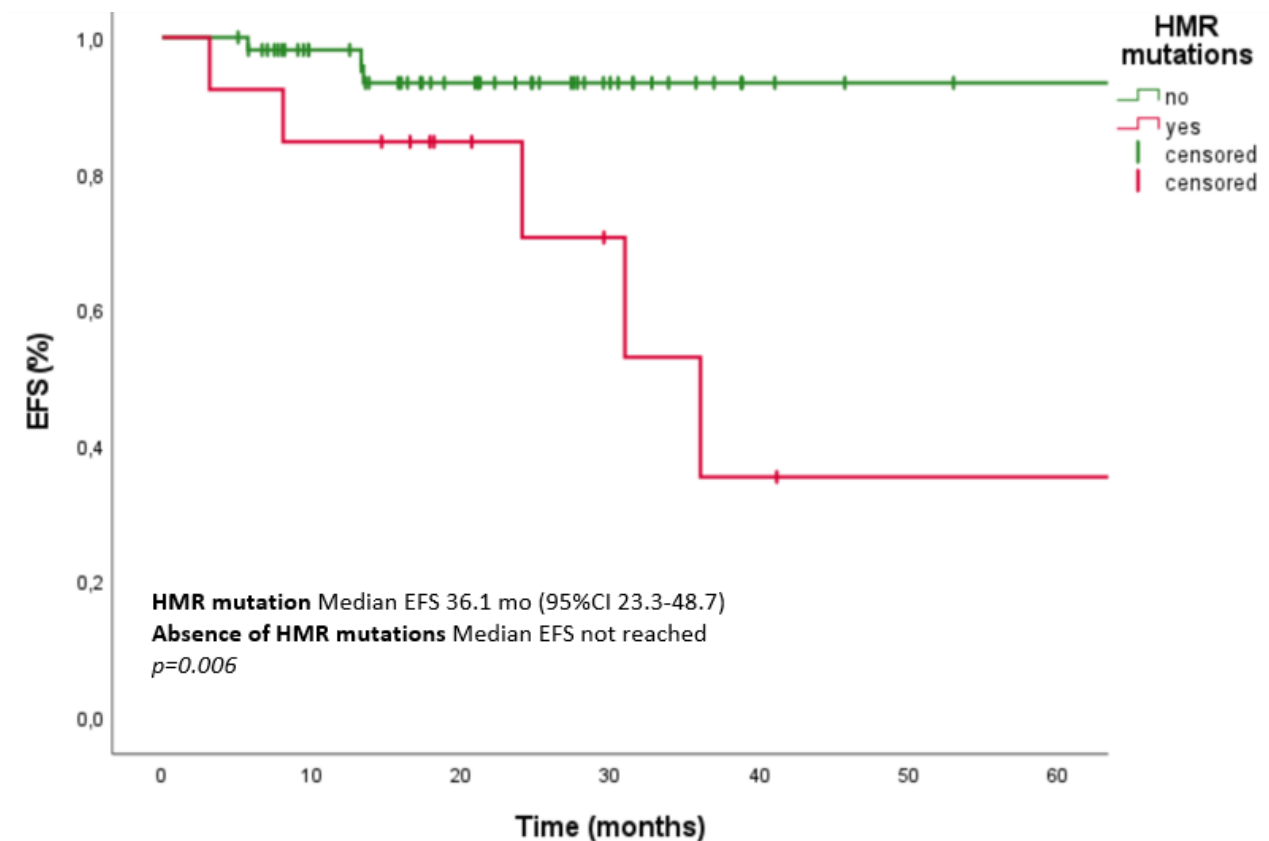
Figure 28. EFS according to *ASXL1* mutation status in all patients
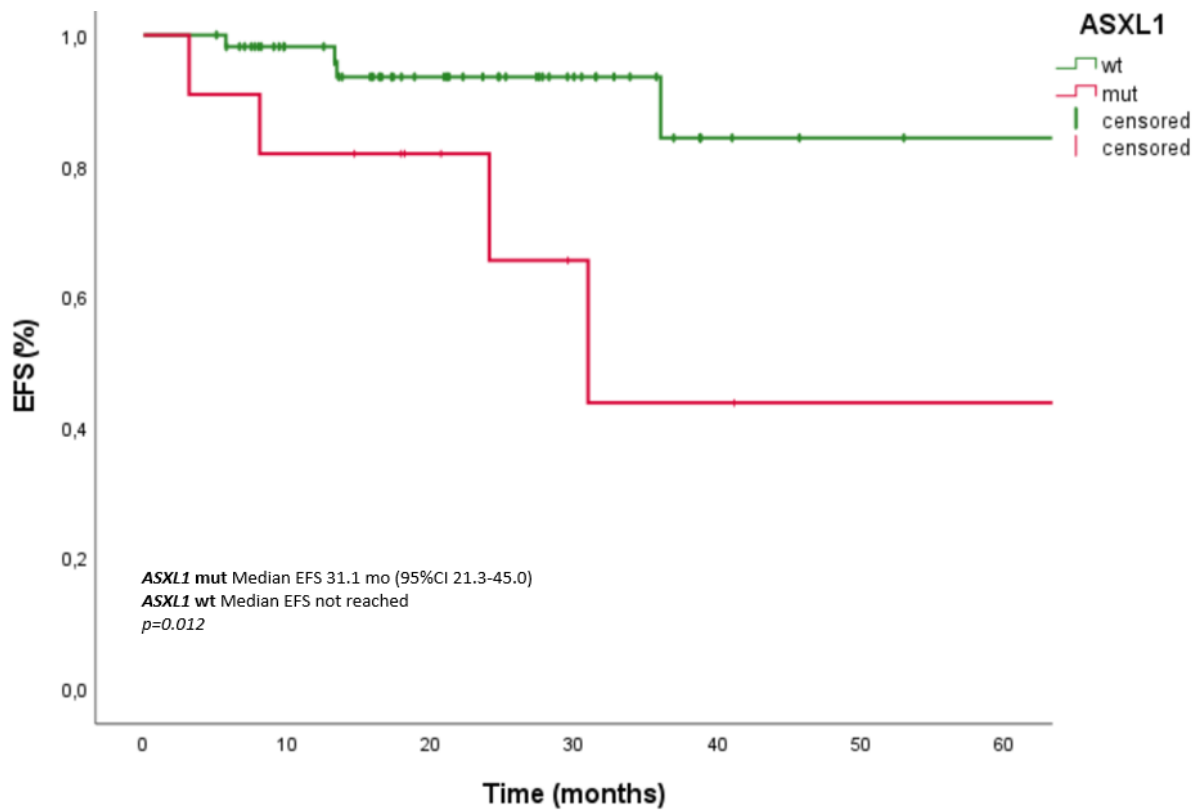
Figure 29. EFS according to the number of non-driver mutations
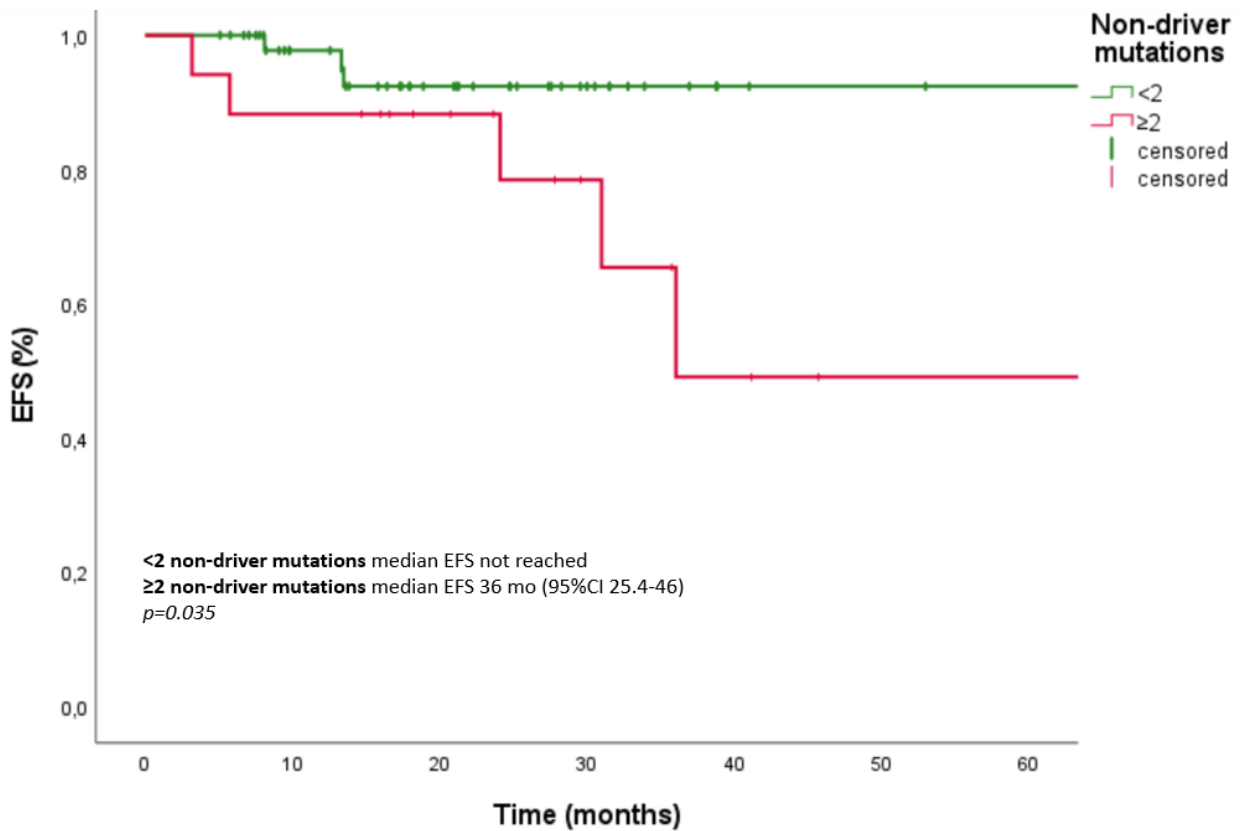
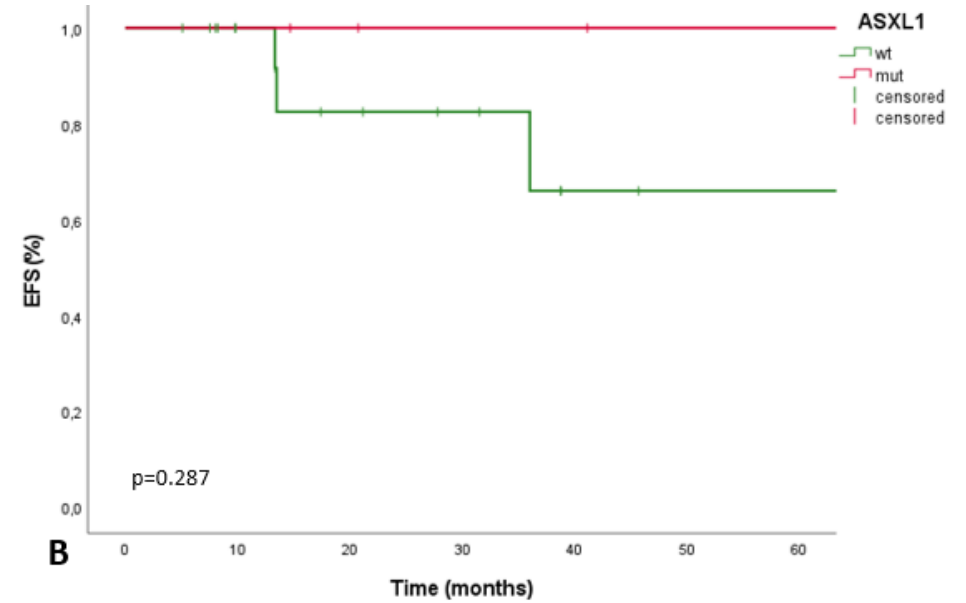**Figure 30. EFS according to *ASXL1* mutation status in PMF (A) and SMF (B) patients**
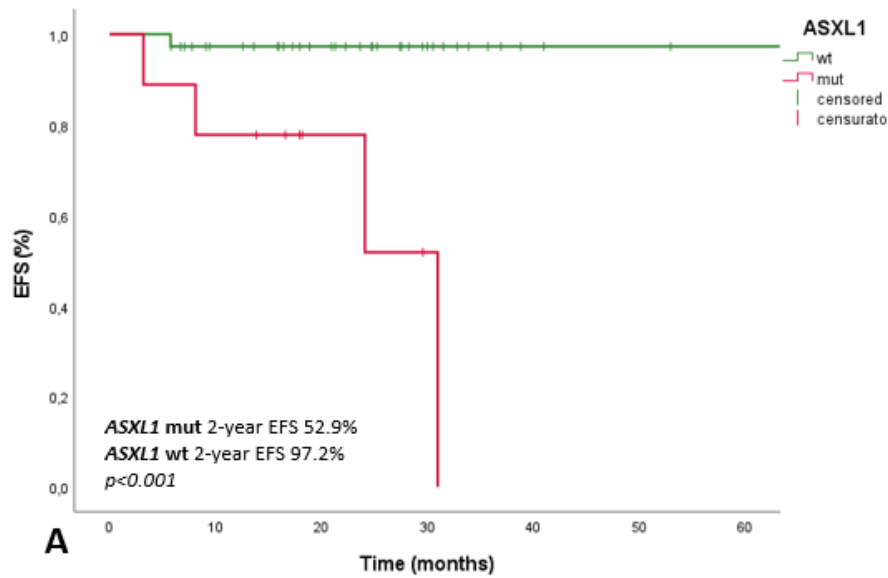

Figure 31. EFS according to the presence of HMR mutations in PMF (A) and SMF patients (B)
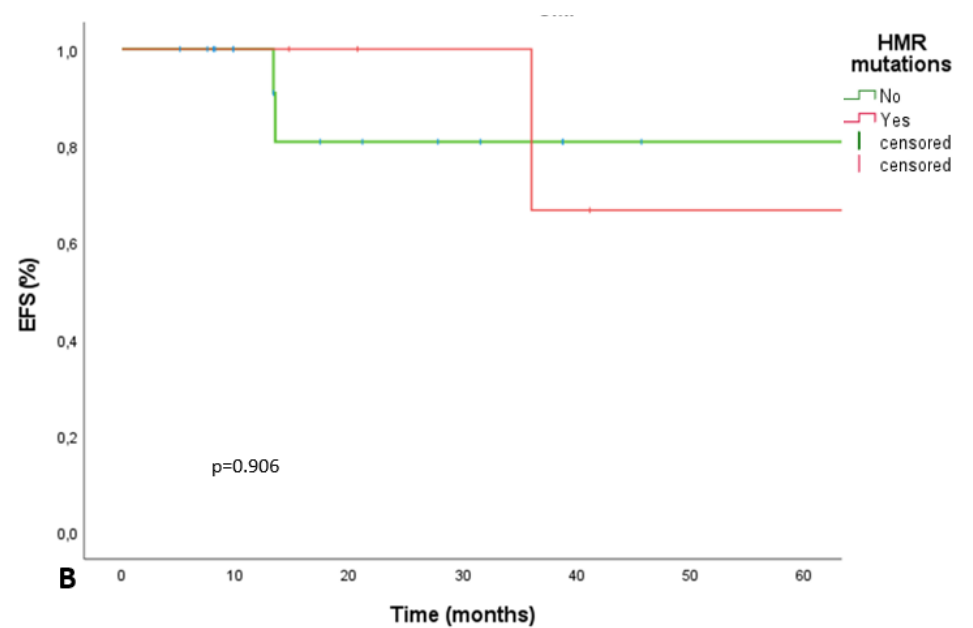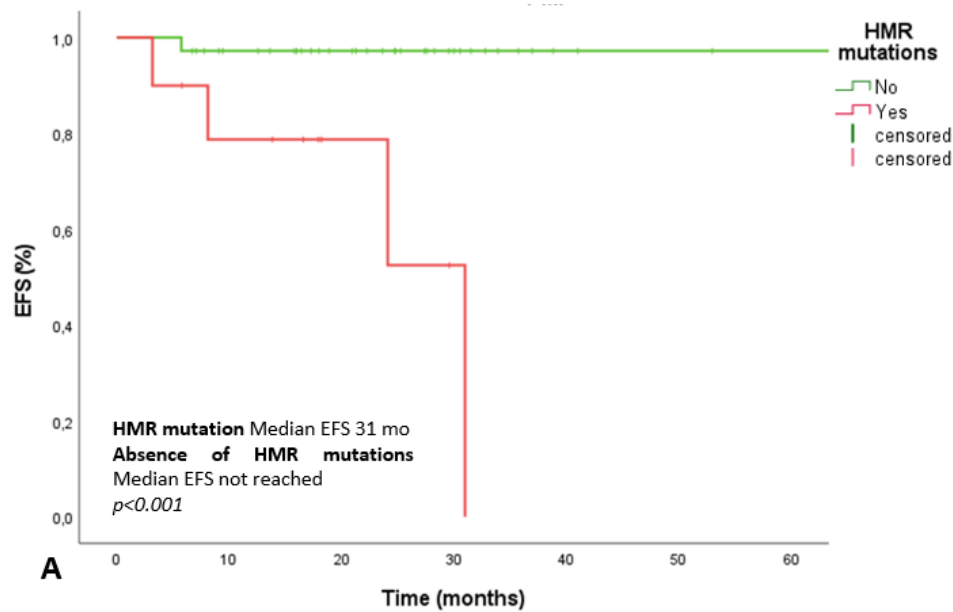
Figure 32. EFS according to the number of non-driver mutations in PMF (A) and SMF patients

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