Genomic Profiling of a Randomized Trial of Interferon-α versus Hydroxyurea in MPN Reveals Mutation-Specific Responses

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Abstract: Background Although somatic mutations influence the pathogenesis, phenotype, and outcome of myeloproliferative neoplasms (MPN), little is known about their impact on molecular response to cytoreductive treatment. Methods We performed targeted next-generation sequencing (NGS) on 202 pre-treatment samples obtained from patients with MPN enrolled in the DALIAH trial (randomized controlled phase III clinical trial, NCT01387763) and 135 samples obtained after 24 months of therapy with recombinant interferon-alpha (IFNα) or hydroxyurea (HU). The primary aim was to evaluate the association between complete clinicohematologic response (CHR) at 24 months and molecular response through sequential assessment of 120 genes using NGS. Results Among JAK2-mutated patients treated with IFNα, those with CHR had a greater reduction in the JAK2 variant allele frequency (VAF) (median 0.29 to 0.07; p<0.0001) compared with those not achieving CHR (median 0.27 to 0.14; p<0.0001). In contrast, the CALR VAF did not significantly decline in neither those achieving CHR nor those not achieving CHR. Treatment-emergent mutations in DNMT3A were observed more commonly in patients treated with IFNα compared with HU, p=0.04. Furthermore, treatment-emergent DNMT3A-mutations were significantly enriched in IFNα treated patients not attaining CHR, p=0.02. A mutation in TET2, DNMT3A, or ASXL1 was significantly associated with prior stroke (age-adjusted OR=5.29 [95% CI, 1.59-17.54]; p=0.007) as was a mutation in TET2 alone (age-adjusted OR=3.03 [95% CI, 1.03-9.01]; p=0.044). Conclusion At 24 months, we found mutation-specific response patterns to IFNα: (1) JAK2- and CALR-mutated MPN demonstrated distinct molecular responses and (2) DNMT3A-mutated clones/subclones emerged on treatment.

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Title Page

Title
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Short title (for running head)
Genomic Profiling of a RCT of IFNα vs HU in MPN

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Key Points

- Treatment with IFNα was associated with distinct molecular responses in patients with JAK2-mutated as compared with CALR-mutated MPN.
- Amongst patients treated with IFNα who did not achieve CHR, DNMT3A-mutations emerged more frequently than non-DNMT3A-mutations.
Abstract

Background
Although somatic mutations influence the pathogenesis, phenotype, and outcome of myeloproliferative neoplasms (MPN), little is known about their impact on molecular response to cytoreductive treatment.

Methods
We performed targeted next-generation sequencing (NGS) on 202 pre-treatment samples obtained from patients with MPN enrolled in the DALIAH trial (randomized controlled phase III clinical trial, NCT01387763) and 135 samples obtained after 24 months of therapy with recombinant interferon-alpha (IFNα) or hydroxyurea (HU). The primary aim was to evaluate the association between complete clinicohematologic response (CHR) at 24 months and molecular response through sequential assessment of 120 genes using NGS.

Results
Among JAK2-mutated patients treated with IFNα, those with CHR had a greater reduction in the JAK2 variant allele frequency (VAF) (median 0.29 to 0.07; p<0.0001) compared with those not achieving CHR (median 0.27 to 0.14; p<0.0001). In contrast, the CALR VAF did not significantly decline in neither those achieving CHR nor those not achieving CHR. Treatment-emergent mutations in DNMT3A were observed more commonly in patients treated with IFNα compared with HU, p=0.04. Furthermore, treatment-emergent DNMT3A-mutations were significantly enriched in IFNα treated patients not attaining CHR, p=0.02. A mutation in TET2, DNMT3A, or ASXL1 was significantly associated with prior stroke (age-adjusted OR=5.29 [95% CI, 1.59-17.54]; p=0.007) as was a mutation in TET2 alone (age-adjusted OR=3.03 [95% CI, 1.03-9.01]; p=0.044).

Conclusion
At 24 months, we found mutation-specific response patterns to IFNα: (1) JAK2- and CALR-mutated MPN demonstrated distinct molecular responses and (2) DNMT3A-mutated clones/subclones emerged on treatment.
Introduction

Philadelphia chromosome-negative chronic myeloproliferative neoplasms (MPN) comprise essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF), including pre-fibrotic myelofibrosis (Pre-MF). MPNs are clonal hematopoietic neoplasms characterized by excessive proliferation of mature hematopoietic cells from one or more of the myeloid lineages.\textsuperscript{1,2} The diseases are associated with an increased risk of thrombohemorrhagic events and reduced life expectancy compared with the general population.\textsuperscript{3,4} ET and PV may progress into post-ET and post-PV myelofibrosis, and all disease entities may transform into secondary acute myeloid leukemia (sAML), which has a dismal prognosis.\textsuperscript{5}

The majority of MPNs are driven by somatic mutations in JAK2, CALR, or MPL that arise in the hematopoietic stem cell compartment (ie MPN phenotypic driver mutations).\textsuperscript{6} All three MPN phenotypic driver mutations lead to uncontrolled myeloproliferation by constitutive activation of the JAK-STAT signal transduction pathway through ligand-independent activation and hypersensitivity of type I cytokine receptors.\textsuperscript{7} Approximately 95-97\% of patients with PV and 50-60\% of patients with ET or PMF harbor a point-mutation in exon 14 of the JAK2 gene.\textsuperscript{8-11} The remaining 2-3\% of PV patients carry mutations in JAK2 exon 12.\textsuperscript{12} CALR or MPL mutations are present in the majority of JAK2-negative ET and PMF patients. Approximately 10\% of patients with MPN carry none of the three phenotypic driver mutations and are referred to as "triple-negative".\textsuperscript{13,14}

The emergence of next-generation sequencing (NGS) has expanded insights into the molecular complexity of MPN, and more than 50 genes have been reported to be recurrently mutated.\textsuperscript{15} Mutations outside of JAK2, CALR, and MPL (ie concomitant somatic mutations) are observed in >50\% of patients with MPN, and increasing numbers are observed with disease progression.\textsuperscript{16,17} The most common classes of concomitant mutations consist of genes involved in DNA methylation (TET2, DNMT3A, IDH1, IDH2), chromatin modification (ASXL1, EZH2), RNA splicing (SRSF2, U2AF1, SF3B1, ZRSR2), signaling pathways (LNK/SH2B3, CBL, NRAS, KRAS, PTPN1), transcription factors (RUNX1, NFE2), and DNA damage response/stress signaling (TP53, PPM1D).\textsuperscript{7} These mutations may precede the acquisition of the phenotypic driver mutation or occur subsequently in the same or a
different clone. Concomitant mutations may contribute to phenotype and are often associated with disease progression and inferior survival. Furthermore, the presence of specific concomitant mutations, as well as the total number and order of acquisition, influence prognosis.

Internationally, the most widely used first-line cytoreductive therapy in patients with high-risk ET or PV is hydroxyurea (HU). HU effectively reduces the elevated peripheral blood counts and the risk of thrombosis. However, conflicting evidence exists regarding the potential of HU to induce a continuous reduction of the JAK2V617F-mutated clone. In contrast, recombinant interferon-alpha (IFNα), which has been used off-label for the treatment of MPN for more than three decades, has been associated with molecular responses in JAK2V617F-mutated MPN. A subset of patients achieve molecular remissions and normalization of the bone marrow after long-term treatment, which may be sustained in a minority of patients even after treatment discontinuation, an effect never observed for HU.

Increasing knowledge on the complex molecular landscape in MPN has enabled more accurate personalized prediction of outcome and improved clinical decision-making, particularly in myelofibrosis. However, the predictive role of somatic mutations regarding response and resistance to cytoreductive therapy remains unclear. To address this question, we performed serial genomic profiling on patients enrolled in the DALIAH trial, which to our knowledge is the largest randomized controlled phase III trial of HU vs IFNα in patients with newly diagnosed MPN.

Methods

Trial design

Genomic profiling by NGS was performed in 202 pre-treatment samples and 135 samples obtained after 24 months from patients enrolled in the DALIAH trial. The DALIAH trial was an investigator-initiated, open-label, randomized controlled, parallel design, clinical phase III trial (ClinicalTrials.gov Identifier: NCT01387763). The study was approved by the Danish Regional Science Ethics Committee and the Danish Medicines Agency and conducted in compliance with the Declaration of
Helsinki and Good Clinical Practice. All study participants provided written informed consent before entering the trial.

Patients aged ≥ 18 years with a diagnosis of ET, PV, Pre-MF, or PMF according to the WHO 2008 criteria and evidence of active disease regardless of risk-group were eligible to be enrolled. Detailed inclusion and exclusion criteria are provided in Supplementary Information pp. 5-6. Patients > 60 years of age were randomly allocated (1:1:1) to receive HU, IFNα-2a, or IFNα-2b whereas patients ≤ 60 years of age were randomly allocated (1:1) to receive IFNα-2a or IFNα-2b. Treatment dose was modified based on efficacy and toxicity according to predefined dose levels (Table S1-S2). Clinicohematologic response assessment was performed by central review according to modified 2009 European Leukemia Net (ELN) (ET, PV, Pre-MF) and 2005 European Myelofibrosis Network (EUMNET) criteria (PMF).

NGS analysis
Genomic profiling comprised targeted NGS of 120 myeloid malignancy-associated genes and 1609 informative single nucleotide polymorphisms (SNPs) on chromosome 9p. Detailed information on the sequencing, including a list of sequenced genes and genomic coordinates of all target regions, is provided in the Supplementary Information pp. 10-11 and Table S6-S8.

Statistical methods
Statistical methods are presented in Supplementary Information pp. 11-12.

Results

Clinical characteristics at baseline
NGS was performed on 202 pre-treatment samples from patients randomly allocated to treatment with HU (38) or IFNα-2a (164), and 135 samples were obtained 24 months after initiation of therapy (HU: 34 or IFNα: 101) (Figure 1). Seventy-two patients (36%) had ET, 89 (44%) had PV, 16 (8%) had Pre-MF and 25 (12%) had PMF (Table 1). The median age was 62 years (range, 20-88), and 112 (55%) were male. Thirty-nine (19%) patients had experienced prior major thrombosis including 17 (8%) with prior stroke (ET: 4/72 (6%), PV: 10/89 (11%), Pre-MF: 1/16 (6%), PMF: 2/25 (8%). Twenty-
one (10%) patients received HU from screening and until random allocation in the study due to major thrombosis at diagnosis or platelet count > 1000 x 10^9/L at screening. The median time from screening to randomization in these patients was 21 days (range, 3-45). Prior phlebotomy was performed in 90 (45%) patients with a median number of three phlebotomies in each patient (range, 1-29). Due to the design of the study median age was higher in patients allocated to HU (68 years (interquartile range (IQR), 64-71)) compared with IFNα (59 years (IQR, 46-67)). Baseline demographics and clinical characteristics are presented in Table 1, and Table S9-S10.

### Somatic mutations at baseline

Somatic mutations in 34 genes were detected by NGS in 191 (95%) patients at baseline. MPN phenotypic driver mutations were present in 92% of the patients: **JAK2** (74% - **JAK2**V617F=73%, **JAK2** exon 12=1%), **CALR** (14% - type 1=11%, type 2=3%), or **MPL** (5%) (Figure 2 and Table S11). No somatic mutations were detected in 11 patients (5%), 1 mutation was detected in 88 patients (44%), 2 mutations in 55 patients (27%), and ≥3 mutations in 48 patients (24%). The number of mutations was significantly different based on diagnosis (mean number of mutations in ET: 1.6 (SD=1.5), PV: 2.0 (SD=1.3), Pre-MF: 2.1 (SD=1.2), PMF: 1.9 (SD=1.9)). Although the presence of phenotypic driver mutations is usually considered mutually exclusive, we found coexistence of **JAK2**V617F and **MPL** mutations in three patients (1%). Sixteen (8%) patients (ET: 12, PV: 2, Pre-MF: 1, PMF: 1) were triple-negative for **JAK2**, **CALR**, and **MPL** mutations. The median **JAK2** VAF at baseline was 0.25 (range, 0.01-0.94), and **JAK2** uniparental disomy (**JAK2**-UPD) was observed in 28%. The median **JAK2** VAF was significantly higher among patients with **JAK2**-UPD (0.48 (IQR, 0.35-0.68)) compared to those without **JAK2**-UPD (0.15 (IQR, 0.09-0.26), p<0.0001). The most frequent concomitant mutations at baseline affected three genes: **TET2** (24%), **DNMT3A** (16%), and **ASXL1** (10%). Spliceosome gene mutations were found in 4% (**SF3B1**: 6, **SRSF2**: 2, **U2AF1**: 1, **ZRSR2**: 1), and mutations involving RAS/MAPK signaling, including **CBL**, **KRAS**, **NRAS**, **NF1**, **PTPN11**, and **RIT1**, were detected in 6% (Figure 2).

### Association between somatic mutations and clinical characteristics at baseline

At baseline, mutations in **JAK2** were detected in 98% of patients with PV and 53%, 69%, and 56% of patients with ET, Pre-MF, and PMF, respectively. **JAK2**-UPD was most commonly found in patients
with PV (54%) (Figure 2), where it was significantly associated with higher hemoglobin (p=0.0003), higher hematocrit (p<0.0001), higher neutrophil count (p=0.039), and lower platelet count (p<0.0001) compared with PV patients without JAK2-UPD (Table S12). JAK2-UPD was not detected in any patients with ET. Among patients with ET, Pre-MF, and PMF, patients with ET were more likely to present with triple-negative disease (ET: 18%, PV: 2%, Pre-MF: 6%, PMF: 4%; p=0.007), which was significantly associated with younger age compared with patients harboring one of the three phenotypic driver mutations (median 44 years vs 64 years; p=0.006). Among patients with ET, Pre-MF, and PMF mutated CALR was significantly associated with higher platelet count (p=0.004) or elevated LDH (p=0.0008) compared to patients with JAK2 (+/- MPL)-mutated MPN or patients with triple-negative MPN (Table S13).

The most frequent concomitant mutations (ie in TET2, DNMT3A, ASXL1, RAS/MAPK signaling, and RNA splicing genes) among all MPN subtypes were detected in both JAK2-mutated and JAK2-wildtype (WT) patients. However, co-existence of ASXL1 was significantly associated with JAK2, being present in 13% of JAK2-mutated patients compared with 2% of JAK2-WT patients (p=0.029) (Table S14). Mutations in TET2, DNMT3A, or ASXL1 were significantly associated with older age (≥60 years) (54% vs 26%; p<0.0001) as well as with a history of major thrombosis (OR=2.11 [95% CI, 1.04-4.37]; p=0.038; age-adjusted OR=1.96 [95% CI, 0.94-4.12]; p=0.073) and in particular prior stroke (OR=5.21 [95% CI, 1.64-16.67]; p=0.005; age-adjusted OR=5.29 [95% CI, 1.59-17.54]; p=0.007) compared with patients without these mutations. Also, TET2 alone was significantly associated with prior stroke (age-adjusted OR=3.03 [95% CI, 1.03-9.01]; p=0.044). No other significant baseline associations were detected between clinical characteristics and baseline mutational status.

**Treatment discontinuation within 24 months**

At 24 months, 40% of all patients had discontinued study medication (Table S15). The most frequent reason for treatment discontinuation across all treatment groups was treatment-related toxicity; HU: 8%, IFNα-2a: 30%, and IFNα-2b: 38%. One patient with CALR-positive PMF and a history of chronic obstructive pulmonary disease died from pneumonia after approximately 17 months on treatment with IFNα-2b. None of the patients transformed to post-ET/PV myelofibrosis or sAML.
Clinicohematologic response at 24 months

At 24 months, 121 patients were on study medication and eligible for clinicohematologic response assessment. Missing data made response evaluation impossible in three of these patients. CHR was achieved in 21% [95% CI, 10-37%] treated with HU and 26% [95% CI, 19-33%] treated with IFNα (IFNα-2a: 30%, IFNα-2b: 21%), p=0.68 (Figure 3A and Table S16). Median time to CHR was 5.7 months (IQR, 1.8-10.5) for HU, 4.9 months (IQR, 2.1-8.9) for IFNα-2a, and 6.0 months (IQR, 1.8-10.1) for IFNα-2b. Of note, 31 (19%) patients allocated to IFNα received either pre-treatment with HU (n=17) and/or combination treatment with IFNα and HU (n=28) within 24 months after treatment allocation (Table S17). At clinicohematologic response assessment at 24 months, seven patients (HU: n=1; IFNα: n=6) received combination treatment. The median duration of combination treatment among these patients was 14.3 months (range, 6.2-18.4). Two were in CHR, one was not evaluable due to missing data, and four were non-responders.

Somatic mutations on serial sampling

NGS was performed in 135 patients at 24 months, including 113 of 121 patients eligible for clinicohematologic response assessment (HU: 32; IFNα: 84) and in 19 who had discontinued study treatment (HU: 2; IFNα: 17) (Figure S2). Phenotypic driver mutations remained detectable by NGS at 24 months in all patients. JAK2 VAF decreased in 94% of the patients treated with IFNα and in 75% treated with HU (p=0.01). The median absolute JAK2 VAF reduction (baseline to 24 months) was significantly greater in patients treated with IFNα (HU: 0.05 vs IFNα: 0.11; p=0.005). The change in CALR VAF with treatment was more heterogeneous. The CALR VAF decreased in 80% of patients allocated to HU and 78% allocated to IFNα (p=0.99) (median VAF reduction: HU: 0.02 vs IFNα: 0.04; p=0.63) (Table S16). Among patients treated with IFNα, those with JAK2-UPD had a greater absolute JAK2 VAF reduction (median 0.49 to 0.17) compared to those without JAK2-UPD (median 0.15 to 0.08), p<0.0001. No significant reduction in JAK2 VAF was observed among patients with JAK2-UPD treated with HU (median 0.44 to 0.30) than those without JAK2-UPD (0.22 to 0.08), p=0.76.

Mutations were detected in 30 genes at 24 months, including 3 not observed at baseline (EP300, IDH2, PHF6) (Figure S2). Thirty-eight treatment-emergent mutations were detected in 32 patients (HU: 14; IFNα: 18), of which 4 patients had discontinued treatment.
DNMT3A was the most frequent treatment-emergent mutation (n=15, 39%), followed by TET2 (n=4, 11%), ASXL1 (n=3, 8%), PPM1D (n=3, 8%), and TP53 (n=3, 8%) (Figure 4A and Table S18). The VAF of treatment-emergent mutations was low (median 1.5%), and they primarily occurred in JAK2-positive patients (97%) (Figure 4B). The NGS platform enabled simultaneous evaluation of (1) the molecular response of MPN phenotypic driver mutations, (2) JAK2-UPD at 9p, and (3) detection of treatment-emergent mutations in any of > 100 genes assessed, allowing us to uncover the complexity of molecular responses (Figure 4C-D). Treatment-emergent mutations in DNMT3A were more commonly observed in patients treated with IFNα (11/18, 61%) than HU (3/14, 21%), p=0.046. In contrast, treatment-emergent mutations in PPM1D or TP53 were more common in patients who received HU (5/14, 36%) compared with IFNα (1/18, 6%), p=0.06 (Figure S3).

Association between somatic mutations and complete clinicohematologic response on serial sampling
The probability of CHR at 24 months was not associated with JAK2 (p=0.27), JAK2-UPD (p=0.35), or CALR (p=0.10) baseline mutational status or concomitant mutations in DNMT3A, TET2, or ASXL1 (p=0.40) in the entire cohort or when stratifying by treatment group (HU vs IFNα). Analysis for associations with other concomitant mutations was not feasible due to their low frequency in the cohort. CHR at 24 months was obtained in 34/150 (23%) JAK2-mutated patients, 11/29 (37%) CALR-mutated, and in 18/84 (21%) patients with DNMT3A, TET2, or ASXL1 mutations. The JAK2 VAF declined significantly in patients randomized to HU achieving CHR (median 0.25 to 0.08; p=0.03) but not in those not achieving CHR (median 0.30 to 0.26; p=0.10). Among JAK2-positive patients randomized to IFNα, those attaining CHR had a greater reduction in the JAK2 VAF (median 0.29 to 0.07; p<0.0001) compared with patients who did not achieve CHR (median 0.27 to 0.14; p<0.0001) (Figure 5A). In contrast, the mutant CALR VAF did not significantly decline in either those achieving CHR during treatment with IFNα (median 0.17 to 0.13; p=0.078) or those not achieving CHR (median 0.21 to 0.17; p=0.066) (Figure 5B). Of note, only 18 CALR-positive patients allocated to IFNα were evaluable for response at 24 months. None of the 5 CALR-positive patients allocated to HU achieved CHR.

We divided the patients available for clinicohematologic assessment and serial sampling (n=113) into two groups: (1) those in whom no treatment-emergent mutations were detected (HU: n=18;
IFNα: n=68) (Figure 5C) and (2) those in whom treatment-emergent mutations were detected (HU: n=13; IFNα: n=14) (Figure 5D). We further divided the latter group into those in whom DNMT3A-mutations were detected (HU: n=2; IFNα: n=9) (Figure 5E) and those in whom non-DNMT3A treatment-emergent mutations were detected (HU: n=11; IFNα: n=5) (Figure 5F). Within the group in whom no treatment-emergent mutations were detected, significantly more patients treated with IFNα achieved CHR (35/68, 51%) compared with patients treated with HU (4/18, 22%), p=0.034 (Figure 5C). Of 27 patients with treatment-emergent mutations at 24 months and available for response assessment, 19 (70%) failed to achieve CHR (HU: 10/13, 77%; IFNα: 9/14, 64%), p=0.68 (Figure 5D). We found that treatment-emergent DNMT3A-mutations were significantly enriched among patients treated with IFNα failing to achieve CHR (8/9, 89%) compared with treatment-emergent non-DNMT3A-mutations (1/5, 20%), p=0.02 (Figure 5E-F). Among patients randomized to HU, the 2 patients with treatment-emergent DNMT3A-mutations did not obtain CHR compared with CHR in 3 of 11 patients (27%) with treatment-emergent non-DNMT3A mutations (Figure 5E-F).

Discussion

To determine the impact of molecular genetics on response to front-line cytoreductive therapy in MPN, we performed sequential molecular profiling on samples obtained from patients enrolled in the DALIAH trial, a randomized controlled phase III clinical trial of IFNα versus HU in newly diagnosed patients with MPN.

To enable detailed molecular profiling, we first developed a custom targeted NGS assay encompassing 120 myeloid malignancy-associated genes. We found a significant age-independent association between the presence of a mutation in TET2, DNMT3A, or ASXL1 at baseline and a history of stroke, which remained significant for mutated TET2 alone. Also, we found an association between the presence of a TET2, DNMT3A, or ASXL1 mutation and a history of major thrombosis. However, this did not retain significance when adjusted for age. Previous studies have found an age-independent association between the presence of one or more mutations in TET2, DNMT3A, or ASXL1 and thrombotic events in PV, which was retained for the presence of a TET2-mutation alone.43 However, an association between TET2, DNMT3A, or ASXL1 mutations and thrombosis in
PV was not found in earlier studies. A novel feature of the NGS assay was the ability to determine the presence of JAK2-UPD on 9p, allowing us to distinguish patients who were heterozygous for the JAK2 mutation from those who were homozygous. This is particularly informative in patients with a JAK2 VAF < 50%. We incorporated JAK2-UPD into our analysis of the molecular response, and by combining sequential mutational and JAK2-UPD analyses, we were able to uncover distinct treatment responses in independent clones/subclones in individual patients (discussed below).

In terms of treatment response, we first focused our attention on the two most common MPN phenotypic driver mutations, JAK2 and CALR. We found that more patients treated with IFNα had a decrease in the mutant JAK2 VAF than patients treated with HU. Further, the median mutant JAK2 VAF reduction was significantly greater among patients treated with IFNα than with HU. In contrast, there was no difference in the magnitude of decrease in mutant CALR VAF in patients treated with HU than IFNα, and the median reduction in mutant CALR VAF was < 5% for both HU and IFNα. We found that patients with JAK2-UPD treated with IFNα had a greater decrease in JAK2 VAF than JAK2-mutated patients without JAK2-UPD (not seen with HU). This finding is consistent with a small prospective MPN-study (n=33 patients) by Mosca et al., who reported (in abstract form) that hematopoietic stem cells homozygous for mutated JAK2V167F were more effectively targeted by IFNα than heterozygous cells.

We next evaluated the association between CHR at 24 months and molecular response. CHR rates were similar at 24 months between HU and IFNα, which is in accordance with data (presented in abstract form) from the randomized Myeloproliferative Disorders-Research Consortium (MPD-RC) 112 study of high-risk ET or PV comparing HU with IFNα-2a. Interestingly, we found that CHR at 24 months was associated with a significant VAF reduction in JAK2-mutated but not in CALR-mutated patients treated with IFNα. Although reductions in mutant CALR VAF in response to IFNα treatment have been reported in MPN, previous smaller studies, including a recent retrospective study (n=38 patients) reported by Czech et al., have suggested that CALR-mutant MPN cells are less sensitive to IFNα than JAK2-mutated cells. Strengths of our findings on this point include that patients were treated on a large prospective randomized trial (n=202 patients) and that JAK2 and
CALR VAF were assessed simultaneously using the same NGS platform. Limitations include the fact that almost one-third of the CALR-mutant group were patients with PMF (31%), in addition to patients with ET (55%) and Pre-MF (14%), whereas the JAK2-mutant group was composed primarily of patients with PV (59%) and ET (24%), in addition to patients with Pre-MF (7%) and PMF (10%).

We next turned our attention to treatment-emergent mutations. By serial sampling at 24 months, we found 38 treatment-emergent mutations in 32 patients. Notably, approximately half the time a treatment-emergent mutation was detected on serial sampling, the JAK2 VAF was found to have declined by more than 50%, suggesting that the treatment-emergent mutation had arisen independently or was sub-clonal to the JAK2-mutant clone. This finding highlights the importance of not restricting molecular analysis in clinical trials to MPN phenotypic driver genes only.

The gene in which we most commonly identified treatment-emergent mutations was DNMT3A (39%), and we found that treatment-emergent DNMT3A mutations were significantly more prevalent in patients treated with IFNα failing to achieve CHR. DNMT3A mutations have been reported to both precede and follow JAK2V617F acquisition, in addition to arising in independent clones in MPN. As such, these DNMT3A-mutations could reflect either treatment-resistant subclones or genetically unrelated clones that develop in parallel to the phenotypic driver clone.

The methodology we used in this study did not allow us to distinguish whether pre-existing DNMT3A-mutated clones expanded during treatment with IFNα or de-novo DNMT3A-mutations were induced by IFNα. However, we believe it is highly likely that treatment-emergent DNMT3A-mutations were pre-existing at baseline and selected for with IFNα therapy. In agreement with this model, recent studies using ultrasensitive error-corrected sequencing, have found that most adults above 50 years of age have evidence of clonal hematopoiesis, most commonly involving mutations in DNMT3A. In accordance with our finding, Quintas-Cardama et al. found that the acquisition of a DNMT3A mutation was associated with failure to achieve complete molecular remission (CMR) in patients with PV and ET treated with IFNα (n=83). More recently, Stetka et al. reported (in abstract form) that genetic loss of Dnmt3a confers resistance to treatment with IFN in a JAK2V617F-driven MPN mouse model. Clues to the mechanism by which Dnmt3a loss could render hematopoietic stem and progenitor cells (HSPCs) resistant to IFNα are suggested by Jacquelin et al., who found that Dnmt3a loss induced aberrant self-renewal of Jak2-mutant HSPCs and increased
pro-inflammatory signaling due to increased chromatin accessibility.\textsuperscript{56,57} It is important to note that the majority of \textit{DNMT3A} mutations found in MPN (and in this study) are heterozygous missense mutations that do not result in complete loss of \textit{DNMT3A} function. Mutations in \textit{PPM1D} or \textit{TP53} were found more frequently in patients treated with HU, a finding consistent with several earlier reports linking mutations in these genes to chemotherapy exposure in other contexts.\textsuperscript{58–61} However, it is important to note that low allele burden \textit{TP53}-mutations have been associated with older age in chronic phase MPN and randomization to HU was restricted to patients > 60 years in our study.\textsuperscript{62}

In this study, we assessed molecular response at 24 months. In previous studies, the \textit{JAK2} molecular response has been demonstrated to increase gradually with time upon treatment with IFN\textalpha,\textsuperscript{28–36} whereas the molecular response is often transient in patients treated with HU.\textsuperscript{25–27,30} In the recently reported randomized CONTINUATION-PV trial of 257 patients with PV allocated to ropeginterferon alpha-2b (ropeg) or best available therapy, mainly HU, significantly higher \textit{JAK2} molecular responses were observed among patients treated with ropeg after 24 and 36 months of treatment.\textsuperscript{30} Furthermore, the higher \textit{JAK2} molecular response rate in the ropeg arm was even more striking after 48 months and was sustained at 60 months.\textsuperscript{63} This is consistent with the reported durable \textit{JAK2} molecular responses beyond 5 years in patients with ET and PV treated with IFN\textalpha-2a.\textsuperscript{32} Notably, ropeg, which is dosed every 2 weeks, appeared to be well tolerated in the CONTINUATION-PV trial,\textsuperscript{64} in contrast to our study, in which 34\% of the IFN\textalpha-treated patients discontinued study medication for toxicity within 24 months, despite a low-dose regimen.

Although NGS technologies are increasingly used in clinical practice to provide prognostic information and guide treatment decisions in MPN, sequential genomic profiling is not usually performed outside of clinical trials. In terms of counseling patients on the possible molecular consequences of cytoreductive therapy, our findings can be summarized as follows: (1) Co-existing mutations are present at diagnosis in approximately 50\% of patients with ET and PV, (2) Concomitant mutations may be present in the same cell or a different cell than the MPN disease-initiating mutation (ie \textit{JAK2}, \textit{CALR}, or \textit{MPL}), (3) Not all mutations respond in the same way to IFN\textalpha or HU treatment. It is important to acknowledge that we currently have an incomplete
understanding of the clinical significance of concomitant mutations in ET and PV, particularly with respect to treatment. Therefore, additional studies with long follow-up are required to understand the clinical significance of an IFNα-induced reduction in JAK2V617F allele burden and mutations, such as DNMT3A, expanding during IFNα treatment. Since the primary goal of cytoreductive therapy in MPN is to reduce the risk of thrombotic and vascular events, we are not suggesting any immediate change in clinical practice based on our results. However, we suggest several next steps to further advance the understanding of the differential effects of cytoreductive therapy on clonal MPN cells: (1) Aggregate currently available molecular genetic data on patients treated with IFNα to increase statistical power and further validate key findings, (2) Perform sequential NGS analysis in prospective clinical trials of cytoreductive therapy in PV and ET, and correlate early molecular findings with long-term clinical outcomes (ie identify molecular genetic biomarkers that predict clinical outcome), (3) Develop low-cost methodologies to enable sequential molecular genetic analysis as a routine component of MPN clinical care.

Finally, newly emerging data have demonstrated that acquisition of the JAK2V617F-mutation may occur decades before the development of MPN, consistent with a long pre-clinical phase termed JAK2-mutant clonal hematopoiesis (CH). Although not all patients with JAK2-mutant CH develop MPN, it is a clinically relevant entity associated with an increased risk of cardiovascular disease and venous thrombosis. Due to the ability of IFNα to reduce the JAK2-mutated clone, early identification and upfront treatment of individuals with JAK2-mutant CH raises the possibility that IFNα could have the potential to prevent the development of MPN and/or decrease JAK2-mutant CH associated morbidity and mortality. The development of specialized CH clinics to identify such individuals and offer them clinical trials (eg with IFNα) is a recent development in this regard.

In conclusion, we performed comprehensive molecular profiling of patients with newly diagnosed MPN treated with front-line cytoreductive therapy (IFNα vs HU) and identified treatment-specific and mutation-specific patterns of response that have clinical implications.

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Authorship

Contribution:
A.M., H.C.H., and R.C.L designed the study, analyzed and interpreted data, edited the manuscript, and oversaw the study. R.C.L. designed and performed genomic analyses and made figures. T.A. Knudsen collected clinical data, analyzed and interpreted data, made figures, and wrote the 1st version of the manuscript. L.F.O. collected clinical data. D.L.H. constructed the clinical database. D.L.H, L.K., T.S.L, and V.S. analyzed and interpreted data and edited the manuscript. W.D. processed samples and analyzed data, C.L. analyzed data. D.S.N., L.W., K. Stevenson., and T. A. Knudsen performed statistical analysis. D.E.F., D.L.H., J. Starklint, J. Stentoft, K. Stricker., M.F., M.B., M.T., M.T.S., O.W.B., T. A. Kruse, T.A., T.K.K., T.S.L., and U.M.O. performed research. C.J.G. analyzed NGS data, A.N., A.R.T., and B.W. oversaw the generation of NGS data. All authors reviewed the manuscript.

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**Figure Legends**

**Figure 1. Trial flow chart.** NGS was performed on 202 primary MPN samples and 135 samples obtained 24 months after initiation of therapy with either HU or IFNα (IFNα-2a or IFNα-2b). One patient allocated to IFNα died within 24 months.

**Figure 2. Genomic profiling of somatic mutations in baseline samples by NGS (co-mutation plot).** Each column represents one patient (n=202), and the rows represent different somatic mutations. The VAF for each phenotypic driver mutation is color-coded. The frequency of specific somatic mutations is listed on the right border of the figure. Somatic mutations in 34 different genes were detected in 191 (95%) patients, including 92% with MPN phenotypic driver mutations: JAK2: 74%, CALR: 14%, or MPL: 5%. JAK2-UPD was observed in 28% and was significantly associated with PV, p<0.0001 (Kruskal-Wallis test). The most frequent concomitant mutations affected three genes: TET2 (24%), DNMT3A (16%), and ASXL1 (10%). Abbreviations: 9p-UPD: uniparental disomy of chromosome 9p.

**Figure 3. Complete clinicohematologic response at 24 months.** (A) Proportion of patients with complete clinicohematologic response (CHR) over time by treatment group. Median time to CHR was 5.7 months (IQR, 1.8-10.5) for HU, and 4.9 months (IQR, 2.1-8.9) and 6.0 months (IQR, 1.8-10.1) for patients treated with IFNα-2a or IFNα-2b, respectively. The CHR rate reached a maximum after 12 months among patients treated with HU (47%), whereas the CHR rate increased almost gradually over time among patients treated with IFNα-2a or IFNα-2b. (B) Proportion of patients with CHR at 24 months by treatment group. CHR was achieved in 8 of 38 (21%, [95% CI, 10-37%]) patients treated with HU, 25 of 82 (30%, [95% CI, 21-42%]) patients treated with IFNα-2a, and in 17 of 82 (17%, [95% CI, 13-31%]) of patients treated with IFNα-2b. No significant difference in the CHR rate was detected between HU and the two IFNα-groups combined. Patients considered non-evaluable (NE) at 24 months had all discontinued the study therapy to which they were allocated, except 3 patients in whom complete diagnostic workup was not available at 24 months (HU: n=1, IFNα-2a: n=2). Error-bars are 95% CI upper limits.
Figure 4. Treatment-emergent mutations at 24 months. (A) Number of treatment-emergent mutations at 24 months. Thirty-eight treatment-emergent mutations were detected in 32 patients, of which 4 patients had discontinued treatment. Mutations were defined as treatment-emergent if (1) the VAF was below 0.01 in the baseline sample and ≥0.01 in the 24 months sample (n=36) or (2) if the VAF was above ≥0.01 in the baseline sample and had a more than 4-fold increase in the 24 months sample (n=2). The most frequent treatment-emergent mutations were detected in DNMT3A (n=15, 39%), followed by, TET2 (n=4, 11%), ASXL1 (n=3, 8%), PPM1D (n=3, 8%), and TP53 (n=3, 8%). (B) The VAF of treatment-emergent mutations at baseline and post-treatment at 24 months. The median VAF of treatment-emergent mutations was low (median 1.5%) and primarily occurred in JAK2-mutated patients (97%). (C-D) Representative examples of treatment-emergent mutations detected in patients treated with HU (C) and IFNα (D). MPN phenotypic driver mutations are depicted with blue lines, treatment-emergent concomitant mutations with red lines, and other concomitant mutations with black lines. The upper panels of (C) and (D) also show 9p-UPD analysis. In all examples, 9p-UPD is no longer detectable post-treatment at 24 months, which is concordant with the decrease in mutant JAK2 VAF.

Figure 5. Association between somatic mutations and complete clinicohematologic response on serial sampling. (A-B) Molecular response among patients allocated to IFNα at baseline (Pre) and at 24 months (Post) of treatment by complete clinicohematologic response (CHR). (A). Among JAK2-mutated patients, those attaining CHR at 24 months had a greater reduction in the JAK2 VAF (median 0.29 to 0.07; p<0.0001) compared with JAK2-mutated patients who did not achieve CHR (median 0.27 to 0.14; p<0.0001). (B) The CALR VAF did not significantly decline among patients achieving CHR nor among those not achieving CHR at 24 months. The middle horizontal lines indicate the median value, box limits indicate the 5th and 95th percentiles, and whiskers indicate the range. All observations are represented by a dot. (C) Number of patients with no treatment-emergent mutations. Significantly more patients with no treatment-emergent mutations treated with IFNα achieved CHR (35/68, 51%) compared with patients treated with HU (4/18, 22%), p=0.03 (D) Number of patients with treatment-emergent mutations. No difference in the number of patients failing to achieve CHR was observed between patients treated with HU (10/13, 77%) or IFNα (9/14, 64%), p=0.68. (E) Number of patients with treatment-emergent DNMT3A-mutations.
Number of patients with treatment-emergent non-$DNMT3A$-mutations. Treatment-emergent $DNMT3A$-mutations were significantly enriched in patients treated with IFNα failing to achieve CHR (8/9, 89%) compared with treatment-emergent non-$DNMT3A$-mutations (1/5, 20%), $p=0.02$. 

Table 1. Baseline demographics and clinical characteristics by treatment group.

| Patient-related variable | HU n=38 | IFNa-2a n=82 | IFNa-2b n=82 | Total n=202 |
|--------------------------|---------|-------------|-------------|------------|
| **MPN subtype**          |         |             |             |            |
| ET                       | 9 (24)  | 30 (37)     | 33 (40)     | 72 (36)    |
| PV                       | 21 (55) | 34 (41)     | 34 (41)     | 89 (44)    |
| Pre-MF                   | 1 (3)   | 9 (11)      | 6 (7.3)     | 16 (8)     |
| PMF                      | 7 (18)  | 9 (11)      | 9 (11.0)    | 25 (12)    |
| **Age (years) median (range)** |         |             |             |            |
| ≤ 60 years               | 68 (60-80) | 60 (21-88) | 58 (20-81) | 62 (20-88) |
| > 60 years               | 38 (100) | 37 (45)     | 37 (45)     | 112 (55)   |
| **Biological sex**       |         |             |             |            |
| Female                   | 14 (37) | 37 (45)     | 39 (48)     | 90 (45)    |
| Male                     | 24 (63) | 45 (55)     | 43 (52)     | 112 (55)   |
| **History of major thrombosis** |       |             |             |            |
|                          | 6 (16)  | 21 (25)     | 12 (15)     | 39 (19)    |
| **History of prior stroke** | 3 (8)   | 10 (12)     | 4 (5)       | 17 (8)     |
| **Phenotypic driver mutation** |       |             |             |            |
| JAK2*                    | 31 (84) | 62 (80)     | 57 (80)     | 150 (74)   |
| CALR                     | 6 (16)  | 10 (14)     | 13 (17)     | 29 (14)    |
| MPL†                     | 1 (3)   | 4 (6)       | 5 (6)       | 10 (5)     |
| Triple-negative           | 1 (3)   | 4 (5)       | 11 (12)     | 16 (8)     |
| **Disease-related variable** |       |             |             |            |
| Hemoglobin (mmol/L)       | 9.3 (7.9-10.2) | 9.0 (8.3-9.9) | 8.9 (8.1-9.5) | 9.0 (8.2-9.8) |
| Hematocrit (vol %)        | 45 (41-52) | 45 (42-47)  | 43 (40-47)  | 44 (41-49) |
| WBC (× 10⁹/L)             | 9.9 (8.1-11.5) | 8.9 (7.6-11.6) | 9.5 (7.8-12.7) | 9.4 (7.7-11.7) |
| Platelets (× 10⁹/L)       | 664 (552-895) | 712 (480-930) | 615 (484-852) | 667 (502-904) |
| LDH (U/L)                 | 242 (216-288) | 232 (180-296) | 224 (177-294) | 229 (184-294) |
| Splenomegaly on imaging (≥13mm) | 15/30 (50) | 21/50 (42) | 31/60 (52) | 67/140 (48) |
| Disease-related symptoms‡ | 19 (50)  | 51 (62)     | 40 (49)     | 110 (54)   |
| **Pre-treatment**         |         |             |             |            |
| HU                        | 4 (11)  | 10 (12)     | 7 (9)       | 21 (10)    |
| Phlebotomy                | 17 (45) | 34 (41)     | 39 (48)     | 90 (45)    |
1 Data are n (%) or median (IQR) unless otherwise indicated.
2 * Mutated JAK2V617F or JAK2 exon 12 mutation.
3 † Co-existence of mutated MPL and JAK2V617F was detected in three patients.
4 ‡ Constitutional symptoms, microcirculatory disturbances, or pruritus.
Figure 1. Trial flow chart.

Assessed for eligibility (n=877)

Excluded (n=671)
Did not meet inclusion criteria, declined to participate, or excluded for other reason (n=671)

Randomized (n=206)
HU (n=38), IFNa (n=168)

MPN diagnosis refuted by reevaluation (n=2)
Never initiated therapy (n=1)
No sample available for NGS (n=1)

NGS at baseline (n=202)
HU (n=38), IFNa (n=164)

Remained on study therapy at 24 months (n=121)
HU (n=33), IFNa (n=88)

Discontinued study therapy within 24 months (n=81)
HU (n=5), IFNa (n=76)

NGS at 24 months (n=116)
HU (n=32), IFNa (n=84)

NGS at 24 months (n=19)
HU (n=2), IFNa (n=17)
Figure 2. Genomic profiling of somatic mutations in baseline samples by NGS (co-mutation plot).
Figure 3. Complete clinicohematologic response at 24 months.
Figure 4. Treatment-emergent mutations at 24 months.

A

Number of treatment-emergent mutations

B

Variant allele frequency

C

D

JAK2

EZH2

ASXL1

PPM1D

SPYL1

JAK2

SF3B1

ASXL1

DNMT3A

NFI

DNMT3A

DNMT3A

DNMT3A

DNMT3A

DNMT3A

ASXL1

JAK2

NFE2

ASXL1

DNMT3A

ASXL1

DNMT3A

DNMT3A

DNMT3A

JAK2

PPM1D

PPM1D

C81
Figure 5. Association between somatic mutations and complete clinicohematologic response on serial sampling.

A) JAK2

B) CALR

C) No treatment-emergent mutations

D) Treatment-emergent mutations

E) Treatment-emergent DNMT3A-mutations

F) Treatment-emergent non-DNMT3A-mutations