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LETTER TO THE EDITOR

Changes in neutrophil-to-lymphocyte ratio in patients with polycythemia vera treated with ruxolitinib reflect *JAK2* variant allele frequency

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The neutrophil-to-lymphocyte ratio (NLR) has received interest as potential biomarker of diseases where sustained inflammation plays a major role, including cardiovascular diseases and tumors[1]. In cancer, tumor growth and dissemination is facilitated by chronic inflammation promoted by activated neutrophils, while systemic and tumor-infiltrating T-lymphocytes are supposed to counteract cancer development by activating immunosurveillance. Similar processes may occur in the vascular system, resulting in atherosclerosis and vascular occlusion. Capturing balance between factors promoting and inhibiting cancer and atherosclerosis, NLR may serve as simple and reliable prognostic biomarker of such different diseases[1].

In polycythemia vera (PV), a myeloproliferative neoplasm (MPN) where sustained inflammatory condition and abnormal neutrophil count and functions are fueled by uncontrolled activation of JAK/STAT pathway caused by *JAK2*^{V617F} mutation, higher NLR was associated with risk of venous thrombosis[2], reduced survival[3], and response to ropeg-interferon[4]. In a Danish population-based longitudinal analysis, increasing NLR was associated with shorter survival in patients with PV and other MPN, compared to unaffected population[5]. A comparative analysis of PV patients treated with hydroxyurea or phlebotomy (ECLAP study, n=202 each), ropeg-interferon or phlebotomy (LOW-PV trial, n= 63 and 64, respectively), and ropeg-interferon or hydroxyurea (PROUD-PV/CONTINUATION-PV study, n= 127 each), showed unique kinetics patterns of NLR upon different treatments. In fact, NLR resulted unchanged from baseline in patients managed with phlebotomy only and hydroxyurea, in the latter instance owing to parallel decline of neutrophils and lymphocytes, while in ropeg-interferon-treated patients, the reduction of NLR was due to preferential lowering of neutrophils[6]. Recent data indicate that neutrophils in PV express reduced levels of Kruppel-like factor 2 in a *JAK2*^{V617F} VAF-inverse manner, that were largely reverted by pegylated interferon alfa[7]. Furthermore, tissue factor synthesis and activity was described in PV neutrophils, possibly contributing to thrombotic risk[8]. An additional explanation for abnormally increased NLR levels and the differential effects of drugs, may be related to the expression of *JAK2*^{V617F} mutation also in lymphoid lineage, that in preclinical model caused an impaired lymphoid differentiation[9]. Reduction of NLR in patients receiving ropeg-interferon significantly correlated with decrease of *JAK2*^{V617F} VAF, and molecular responses to ropeg-interferon were predictive of improved event-free survival[6, 10]. Also relevant to the interpretation of those findings is the evidence, obtained in animals expressing ruxolitinib resistant mutations, that the action of ruxolitinib was independent of *JAK2*^{V617F} signaling, rather it appeared to be mediated by reduction

of pro-inflammatory cytokines produced by stromal cells and non-malignant hematopoietic cells [11]. Another drug currently used for second-line treatment of PV patients is ruxolitinib, a JAK1 and JAK2 inhibitor[12, 13]. Recent data from the MAJIC study and a real-world cohort have shown that progressive decline of *JAK2*^{V617F} VAF under ruxolitinib treatment correlated with improved overall and event-free survival, particularly myelofibrosis-free survival [14, 15].

The aims of current study were (i) to analyze time-dependent changes of NLR, total leukocyte count (WBC), absolute counts of neutrophils (ANC) and lymphocytes (ALC), in a prospective cohort of patients with PV who received ruxolitinib as second line therapy, and (ii) to exploit correlation of baseline and upon treatment changes of *JAK2*^{V617F} VAF and NLR levels, and their relationships with disease characteristics. The study included forty-one pts with PV who received ruxolitinib, 10 mg BID for a median of 8.7 years (0.8-9.8) because of intolerance (44.2%) or resistance (55.8%) to hydroxyurea; the overall FU was 9.2 years (1.3-14.5). Patients were managed at our Institution according to conventional treatment criteria for PV and following the label of ruxolitinib (10 mg BID), as part of an observational study that allowed serial samples to be prospectively collected for exploratory analyses. The study was approved by Ethic Committee as part of the MYNERVA project (#21267; MYeloid NEoplasms Research Venture AIRC); participants provided a signed inform consent.

The cohort was enriched in patients with advanced disease, as shown by the long disease duration prior to ruxolitinib treatment (median, 6.2 years) and the high *JAK2*V617F VAF (median, 68.3%), with 76% of the patients having greater than 50% VAF, which may have contributed to the relatively high rate of evolution to postPV-MF (Table 1). Ten patients (24.4%) had history of thrombosis prior to treatment. All but 2 patients were still on treatment at the latest time point (4 years). A complete clinical-hematologic response (CHR) and a molecular response (MR), according to the IWG-MRT criteria, was observed in 32 (78%) and 17 (41.5%) patients, respectively. The median NLR value in the whole population at baseline was 5.2 (95% CI, 1.6-25.0); we accordingly divided pts into a high (H-, above the median) and low (L-, below the median) NLR category. Analysis of patients' characteristics at baseline, showed that, when compared to L-NLR patients, those in the H-NLR category had significantly higher median WBC (12.6 *vs* 8.1 x10^9/L; *P*=0.002), due to increased ANC (12.1 *vs* 5.9 x10^9/L; *P*<0.001) while ALC (1.4 *vs* 2.2 x10^9/L; *P*=0.001) was lower. Conversely, hemoglobin level (15.0 *vs* 14.0 g/dL; *P* =0.01) and platelet count (541 *vs* 323.5 x10^9/L; *P*=0.006) was

higher in L-NLR compared to H-NLR category. There was no baseline difference in disease duration, splenomegaly, evolution to PPV-MF and cardiovascular events, between the two patient categories (**Table 1**).

In the entire cohort, NLR value at baseline resulted correlated with *JAK2*^{V617F} VAF (r=0.5, *P*=0.001; Pearson test). Accordingly, *JAK2*^{V617F} VAF (mean±SD) was significantly higher in H-NLR (81.8±13.1%) compared to L-NLR category (54.8±23.8%; *P*<0.001), while the proportion of patients with VAF $\geq$50% was 100% *versus* 52.4% (*P*<0.001), respectively. Furthermore, the correlation between NLR ratio and *JAK2* VAF was maintained during ruxolitinib treatment, with correlation coefficients of 0.60 (*P*=0.004) at 2 years, 0.55 (*P*=0.048) at 3 years, and 0.66 (*P*=0.003) at 4 years. However, after starting ruxolitinib, the changes in NLR observed in the 2 categories differed. In H-NLR patients, the median baseline NLR value of 9.6 (95% CI, 5.3-25.0) decreased to 5.4 (2.7-11.0; *P*=0.002), 5.2 (1.9-8.7; *P*=0.001) and 4.2 (1.3-11.5; *P*=0.001) at 1, 2 and 3 years, remaining steadily stable afterwards (**Figure 1A**). Changes in NLR levels were due to progressive, preferential decrease of ANC (from a median of 12.1 x10^9/L at baseline to 6.7 x10^9/L (-45%; *P* =0.001), 6.4 x10^9/L (-47%; *P*=0.002), and 5.0 x10^9/L (-59%; *P* =0.001) at 1, 2, and 3 years), while ALC remained largely the same, from a baseline of 1.4x10^9/L (0.1-2.8) to 1.35 (1.0-3.0; p=0.5), 1.24 (1.0-3.0; p=0.6) and 1.14 (1.0-5.0; *P*=0.4) **(Figure 1B).** Conversely, in the L-NLR group, no statistically significant modification of NLR was noticed, with values of 3.1x10^9/L (1.6-5.0) at baseline to 2.6 (1.1-8.2; *P*=0.5), 3.0 (1.0-5.0; *P*=0.16) and 3.2 (1.0-7.7; *P*=0.29) at 1, 2 and 3 years, respectively (**Figure 1A, 1B**).

Also changes in *JAK2*^{V617F} VAF followed different patterns in the 2 NLR categories. In the H-NLR category, a baseline VAF value of 81.8±13.1% decreased to 72.0±17.6% (*P*=0.07), 62.1±26.0% (*P*=0.007), 62.5±24.3% (*P*=0.006) and 52.5±27.5% (*P*=0.008) at year 1, 2, 3 and 4, respectively. Conversely in L-NLR group, changes of *JAK2*^{V617F} VAF were not statistically significant at any time point, from a baseline value of 54.8±23.8% to 37.7±24.7% (*P*=0.11), 43.4±30.8% (*P*=0.16), 41.9±34.8% (*P*=0.21) and 45.0±37.4 (*P*=0.36) at year 1, 2, 3 and 4, respectively (**Figure 1C**).

Baseline NLR levels had no significant relationships with CHR, thrombosis, progression to MF, evolution to AML, or death along ruxolitinib treatment, in either NLR categories. However, the decline of NLR at 4 years was associated with lower progression rate to PPV-MF; patients who did not progress (n=25) had statistically significantly reduced NLR levels (from median of 4.6 (range, 1.6-21.0) to 2.8 (1.3-7.0), -30% from baseline) compared to those who progressed (n=16; from 5.8 (1.9-13.0) to 4.9 (1.3-8.4); -16%) (*P*=0.03). Similar findings for *JAK2*^{V617F} VAF, that reduced from a mean

of 62.6±21.4% to 38.4±18.4% (-39%) in those who did not progress compared to a -11% reduction (from 78.8±16.1% to 63.1±22.6%) in those who did (P<0.01).

Overall, these data, with the limitation of small numbers but the value of long follow-up, suggest that higher NLR values in patients with PV are associated with unique clinical characteristics at baseline, including higher *JAK2*^{V617F} VAF, as well as peculiar response profile in response to ruxolitinib treatment. In fact, H-NLR patients showed statistically significant reduction of NLR value, due to preferential decrease of ANC, that were associated with significant decline of *JAK2*^{V617F} VAF decline, unlike patients with lower baseline NLR values. Of interest, we observed significantly greater reduction of NLR, paralleled by similar decline of *JAK2*^{V617F} VAF, in patients who did not progress to post-PV myelofibrosis, compared to those who evolved. However, we acknowledge that observed correlation of NLR with *JAK2*^{V617F} VAF reduction and the rate of myelofibrotic progression, do not establish any causality relationship between them. Given current findings are reminiscent of observations in patients with PV treated with ropeg-interferon in the LOW-PV and PROUD-PV/CONTINUATION-PV study, we propose that monitoring NLR might represent a simple and affordable surrogate test, rather than molecular monitoring, to predict changes of *JAK2* VAF in response to treatment with drugs that may have the potential to reduce mutation burden, as are interferon and ruxolitinib. We surmise that our findings deserve confirmation in other, larger, cohorts of patients with PV treated with ruxolitinib.

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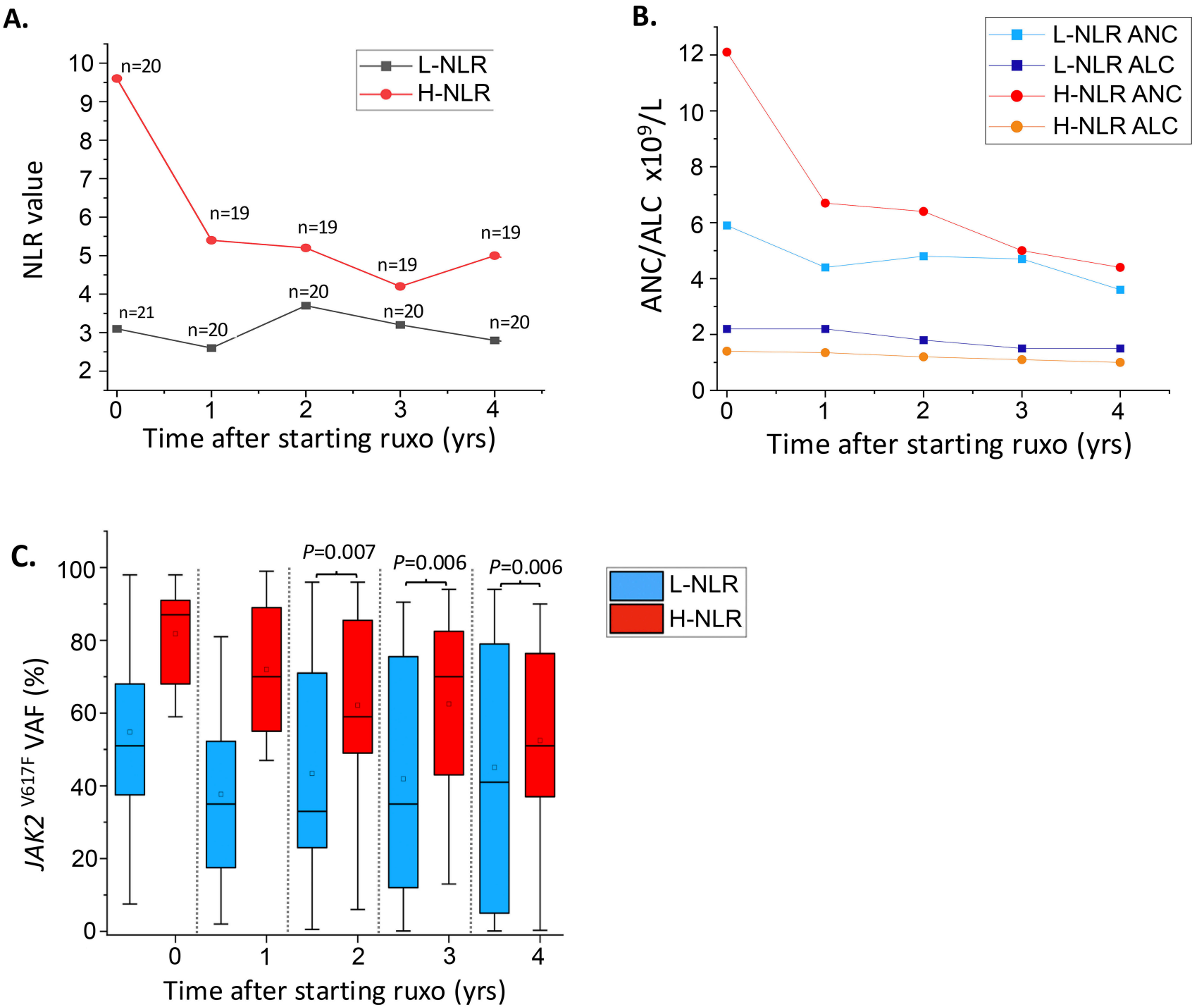
Table 1. Main clinical characteristics at baseline and outcome of patients divided into High-NLR and Low-NLR category.

Variables	All patients *n*=41	High NLR *n*=20	Low-NLR *n*=21	*P*-value
Age in years, median (range)	56.1 (34.6-74.3)	57.9 (34.6-74.3)	55.5 (37.7-71.1)	0.58
Female gender, *n* (%)	21 (51.2)	10 (50)	11 (52.4)	0.88
Hemoglobin g/dl, median (range)	14.7 (12.0-18.7)	14.0 (12.0-17.6)	15.0 (12.6-18.7)	**0.01**
Hematocrit, mean %, (range)	47 (38.0-.57.7)	46.2 (38.0-51.0)	47.9 (39.2-57.7)	**0.002**
Leukocyte count, 10^9/l, median (range)	10.7 (4.8-25.5)	12.6 (6.5-21.6)	8.1 (4.8-25.5)	**0.002**
ANC count, 10^9/l, median (range)	8.8 (9.4-26.3)	12.1 (5.1-26.3)	5.9 (4.0-15.8)	**<0.001**
ALC count, 10^9/l, median (range)	1.6 (0.6-6.5)	1.4 (0.6-2.8)	2.2 (1.0-6.5)	**0.001**
Platelet count, 10^9/l, median (range)	389 (111-898)	323.5 (111-757)	541 (156-898)	**0.006**
*JAK2*V617F VAF, mean % (±SD)	68.3±23.4	81.8±13.1	54.8±23.8	**<0.001**
Patients with *JAK2*V617F VAF $\geq$50%	31 (75.6)	20 (100)	11 (52.4)	**<0.001**
Major thrombosis at or prior to diagnosis, n (%)	10 (24.4)	6 (30.0)	4 (19.0)	0.41
Thrombosis score, n (%) - Low - High	 12 (29.3) 29 (70.7)	 5 (25.0) 15 (75.0)	 7 (33.3) 14 (66.7)	0.56
Palpable splenomegaly, *n* (%)	30 (73.2)	17 (85.0)	13 (61.9)	0.09
Disease duration (years) before starting ruxolitinib, median (range)	6.2 (0.7-22.6)	7.9 (0.7-17.1)	3.83 (0.7-22.6)	0.36
Duration of ruxolitinib therapy, years (range)	8.7 (0.8-14.5)	8.6 (0.8-14.5)	8.0 (1.3-14.3)	0.75
Patients achieving CHR, n (%)	32 (78)	16 (80.0)	16 (76.2)	0.77
Patients achieving MR, n (%)	17 (41.5)	7 (35.0)	10 (47.6)	0.41
Thrombosis, n (%)	2 (4.8)	1 (5.0)	1 (4.7	
Evolution to post-PV MF, n (%)	16 (39.0)	9 (45.0)	7 (33.3)	0.44
Evolution to blast phase, n (%)	2 (4.9)	1 (5.0)	1 (4.8)	0.97
Deaths, n (%)	3 (7.9)	1 (5.0)	2 (9.5)	0.58

Figure legend.

Figure 1. Changes in NLR, absolute neutrophil and lymphocyte counts, and *JAK2* variant allele frequency, during treatment with ruxolitinib. Panel A shows the kinetics of NLR value changes upon 4 years of treatment with ruxolitinib in the category of Low-NLR (L-NLR) and High-NLR (H-NLR), as defined at baseline (see text for definitions). The number of patients available at each time point is indicated. Panel B shows the corresponding changes in absolute neutrophil (ANC) and absolute lymphocyte (ALC) counts in the 2 patient categories. In Panel C, boxes show the levels of *JAK2*^{V617F} variant allele frequency (VAF) in the 2 patient categories at each annual time point. Statically significant differences between L-NLR and H-NLR are shown on the top for each set of data. The time "0" refers to baseline values, before starting ruxolitinib.

Figure 1


A.
NLR value
Time after starting ruxo (yrs)
L-NLR
H-NLR
n=20
n=19
n=19
n=19
n=19
n=21
n=20
n=20
n=20
n=20
B.
ANC/ALC x10^9/L
Time after starting ruxo (yrs)
L-NLR ANC
L-NLR ALC
H-NLR ANC
H-NLR ALC
C.
JAK2 V617F VAF (%)
Time after starting ruxo (yrs)
P=0.007
P=0.006
P=0.006
L-NLR
H-NLR