Circulating megakaryocyte and platelet microvesicles correlate with response to Ruxolitinib and distinct disease severity in patients with Myelofibrosis

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Myelofibrosis (MF) and Essential Thrombocythemia (ET) **are clonal disorders with driver mutations (*JAK2, CALR, MPL*), chronic inflammation and abnormalities in megakaryocyte development and platelet activation.** The absence of the 3 "driver" mutations identifies triple negative (TN) patients. Ruxolitinib (*JAK1/2* inhibitor) reduces splenomegaly and constitutional symptoms in MF. However, over 50% of patients fail to achieve or lose the response over time (Tefferi *et al*, 2015; Vainchenker *et al*, 2018).

Extracellular microvesicles (MVs) are size-heterogeneous small vesicles (100-1000 nm) with pleiotropic effects on cell signalling including immunity and inflammation (Butler *et al*, 2018). Megakaryocyte- and platelet-MVs are the most abundant in peripheral blood (PB). However, while the MVs production by megakaryocytes is based on a constitutive mechanism, only activated platelets can produce CD62P+ MVs (Flaumenhaft *et al*, 2009). High serum levels of MVs have been detected in MF and ET (Caivano *et al*, 2015; Zhang *et al*, 2017).

Circulating MVs as biomarkers of disease/malignancy in MPNs is an open question. Here we investigated: 1) the profile of MVs in MF and ET; 2) whether MVs proportions could be related to severity of MF; 3) the role of inflammation on MVs frequency of MF; 4) the effects of ruxolitinib on MVs in MF.

Firstly, we characterized the circulating megakaryocyte- and platelet-MVs frequency. Comparing patients and healthy donors (HD; **Fig 1a, 1b**), megakaryocyte-MVs were significantly decreased in MF ($p<0.001$) and ET ($p<0.001$). By contrast, platelet-MVs were significantly increased in MF ($p<0.01$) and ET ($p<0.001$). Comparing patients groups, platelet-MVs were significantly increased in ET vs MF ($p<0.01$). No significant differences in megakaryocyte- and platelet-MVs distribution were observed between primary or post-PV/post-ET MF. According to mutation status (**Fig 1c, 1d)**, the megakaryocyte-MVs of the *JAK2*$^{(V617F)}$-($p<0.001$)/*CALR*-($p<0.01$) mutated and TN ($p<0.01$) MF patients were significantly decreased as compared to HD. Conversely, the platelet-MVs were significantly increased in the *JAK2*$^{(V617F)}$-($p<0.001$)/*CALR*-($p<0.05$) mutated MF patients only. Comparing the molecular subtypes, the platelet-MVs of the *JAK2*$^{(V617F)}$-($p<0.05$)/*CALR*-($p<0.05$) mutated patients were significantly increased as compared with the TN counterparts. In ET patients (**Supplementary Fig 2a, 2b**), only the megakaryocyte-MVs of the J*AK2*$^{(V617F)}$-($p<0.05$)/*CALR*-($p<0.05$) mutated patients were significantly decreased as compared to HD. By contrast, the platelet-MVs were significantly increased in *JAK2*$^{(V617F)}$-($p<0.001$)/*CALR*-($p<0.01$) mutated and TN patients ($p<0.05$). **Comparing ET molecular subtypes, no significant differences were observed in megakaryocyte- and platelet-MVs.**

Secondly, we explored the circulating megakaryocyte- and platelet-MVs of MF patients according to the IPSS risk score. Intermediate-2/high IPSS risk patients showed a significant decrease in megakaryocyte-MVs along with a significant increase of platelets-MVs as compared to intermediate 1/low IPSS risk patients

(p<0.05 and p<0.01, respectively) and HD (p<0.001) (**Fig 1e, 1f)**. Comparing IPSS subgroups according to molecular subtypes and HD (**Fig 1g, 1h**), we observed that the megakaryocyte-MVs were significantly decreased in higher risk *JAK2*$^{(V617F)}$*-/CALR*-mutated patients (p<0.001, respectively). Concomitantly, the same group (higher risk *JAK2*$^{(V617F)}$*-/CALR*-mutated patients) presented a higher percentage of platelet-MVs (p<0.001, respectively), suggesting a disease-related specific pattern. Surprisingly, we found a positive correlation between the megakaryocyte-MVs percentages of MF and platelets count (r=0.45; p<0.001; **Fig 2a**), suggesting a role of circulating megakaryocyte-MVs as biomarker of thrombopoiesis. In addition, the percentages of megakaryocyte-MVs of MF were inversely related to splenomegaly (r=-0.39; p<0.01**; Fig 2b**), confirming that a high disease severity is associated with reduced circulating megakaryocyte-MVs. Of note, no correlation was found between platelet-MVs and platelets count or splenomegaly.

Thirdly, despite plasma crucial pro-inflammatory cytokines, Thrombopoietin **and soluble (s)P-selectin** were increased in MF (**Supplementary Table 4**), only IL-6 were inversely related with megakaryocyte-MVs percentages (r=-0.38; p<0.05; data not shown). We can therefore hypothesize that in MF IL-6 inhibits megakaryocyte-MVs production and/or increases their clearance. Conversely, the percentages of the platelet-MVs were positively correlated with the Thrombopoietin **and sP-selectin levels confirming a platelet activation-based mechanism** (r=0.51, p<0.01; **r=0.36, p<0.05, respectively;** data not shown). Consistently, Thrombopoietin-driven platelets activation has been previously described (Kojima *et al*, 1995).

Finally, to investigate whether ruxolitinib therapy may affect circulating MVs, MF patients were studied before and after 6 months of therapy. After 6 months, 12 out of 27 (44%) patients were in spleen response. At baseline, the percentages of megakaryocyte-MVs were significantly decreased as compared with the HD counterparts (spleen responders/non-responders p<0.001, respectively), while platelet-MVs significantly increased (spleen responders/non-responders p<0.001, respectively) (**Fig 2c, 2d**). Importantly, non-responders showed a significantly lower median percentage of megakaryocyte-MVs as compared with the spleen responders counterparts (p<0.05) (**Fig 2c**). To further explore whether megakaryocyte-MVs proportion could be linked to ruxolitinib response, we performed a ROC analysis. A cut-off value of 19.95% of megakaryocyte-MVs was calculated with a specificity of 80%/sensitivity of 72% and discriminated the non-responders (megakaryocyte-MVs < 19.95%). Ruxolitinib therapy, along with a significant decrease of platelet-MVs (p<0.01), promoted the release of megakaryocyte-MVs of spleen responders only (p<0.001) (**Fig 2c, 2d**), restoring the normal megakaryocyte- and platelet-MVs profile (**Fig 2e**).

Interestingly, circulating monocyte- and endothelial-MVs (**Supplementary Fig 3a, 3b**) were significantly increased in MF patients (p<0.05 and p<0.01, respectively). At baseline, monocyte- and endothelial-MVs were not significantly different

between spleen responders and non-responders. Ruxolitinib therapy decreased the endothelial-MVs frequency in spleen responders only ($p<0.05$). A trend, albeit not statistically significant, toward a reduction of the monocyte-MVs was also observed in spleen responders.

Overall, these results demonstrate that distinct abnormalities of circulating megakaryocyte- and platelet-MVs profile are associated to MF and ET and suggest that: 1) platelets activation and abnormal/defective megakaryocytopoiesis may contribute to the increased/decreased proportion of circulating platelet- and megakaryocyte-MVs, respectively; 2) the activated JAK/STAT pathway plays a role in MVs biogenesis/clearance and, ultimately, in communication between megakaryocytes/platelets and the other cells. Additionally, circulating megakaryocyte-MVs may be considered a biomarker of thrombopoiesis in MF. Ruxolitinib therapy normalizes the profile of circulating MVs in spleen responders MF patients only by increasing the megakaryocyte-MVs and decreasing the platelet-MVs. Importantly, a cut-off value of 19.95% of megakaryocyte-MVs discriminates spleen responders and non-responders, demonstrating that circulating megakaryocyte-MVs, as a liquid biopsy assay, may be used as potential tool to predict response to ruxolitinib therapy. Therefore, despite the need to be confirmed in a larger casistic, circulating megakaryocyte/platelet-MVs may have a tissue-specific diagnostic and prognostic role in MF.

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Author contributions
M.B., D.F., D.S. and L.C. contributed to study design, statistical analysis and data interpretation. G.A., N.V. and F.P. managed patients and collected blood samples. M.B., F.R. and D.S. performed microvesicles analysis and data interpretation. E.O. performed molecular analysis. M.B., D.F. and D.S. were responsible for cytokines analysis. D.B. and M.B.R. were involved in statistical analysis. M.B., D.F., D.S., M.R., F.P. and L.C. wrote or contributed to write the manuscript. P.L.T. and M.C. reviewed and corrected the manuscripts. All Authors read and contributed to the final version of the manuscript.

Disclosure of Conflict of interest
The authors declare that they have no conflict of interest.

Supporting information
Additional supporting information may be found online in the Supporting Information section.

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Legend to Figures:

Fig 1. **Circulating megakaryocyte- and platelet-MVs frequency of MF and ET patients.** Megakaryocyte-MVs (MK-MVs; CD61+CD62P-) and platelet-MVs (PLT-MVs; CD61+CD62P+) of MF (n=61), ET (n=20) patients and HD (n=20) are shown in panels **(a)** and **(b)**. Panels **(c)** and **(d)** show the frequency of MK- and PLT-MVs of MF patients according to mutation status (*JAK2*$^{(V617F)}$ n=38; CALR n=11; MPL n=6 and TN n=6) and HD (n=20). Panels **(e)** and **(f)** depict MK- and PLT-MVs frequency of MF patients according to IPSS risk (HR= intermediate 2/high IPSS risk (n=37); LR=intermediate 1/low IPSS risk (n=24)). Frequency of MK- and PLT-MVs of MF patients according to mutation status and IPSS risk is shown in panels **(g)** and **(h)** (*JAK2*$^{(V617F)}$*HR* n=22; *JAK2*$^{(V617F)}$*LR* n=16; CALR HR n=6; CALR LR n=5; MPL HR n=6 and TN HR n=3; TN LR n=3). In addition to individual data, median values and interquartile ranges are shown. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001)

Fig 2. (a, b) Correlation between circulating megakaryocyte-MVs frequency and platelets count or splenomegaly in MF patients. Megakaryocyte-MVs (MK-MVs; CD61+CD62P-) percentages **(a)** positively correlates with platelets count and **(b)** negatively with splenomegaly (Spearman's correlation test). **(c, d, e) Circulating megakaryocyte- and platelet-MVs frequency of MF patients according to ruxolitinib therapy response. (c)** and **(d)** show megakaryocyte-MVs (MK-MVs; CD61+CD62P-) and platelet-MVs (PLT-MVs; CD61+CD62P+) of HD (n=20), spleen responders (SR; n=12) and non-responder (NR; n=15) MF patients before (T0) and after 6 months ruxolitinib therapy (6M). In addition to individual data, median values and interquartile ranges are shown. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001). **(e)** the MK- and PLT-MVs combined profile of HD, spleen responders and non-responders before and after 6 months ruxolitinib therapy is shown (mean ± SEM).