

Absence of JAK2V617F Mutated Endothelial Colony-Forming Cells in Patients With JAK2V617F Myeloproliferative Neoplasms and Splanchnic Vein Thrombosis

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Philadelphia (Ph)-negative myeloproliferative neoplasms (MPN) are acquired hematologic diseases with increased production of mature blood cells. They include polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF). The most frequent molecular abnormality found in Ph negative MPN is JAK2V617F, an activating mutation of *JAK2* which is responsible for constitutive signaling of various cytokine receptors. Arterial and venous thromboses are the main complications of these diseases and are responsible for high rates of morbidity and mortality. Of note there is a disproportionate incidence of thrombosis at unusual sites including splanchnic vein thrombosis.¹ Splanchnic vein thromboses (SVT) involve one or more abdominal veins, the two most frequent are Portal Vein Thrombosis (PVT) and Budd Chiari Syndrome (BCS). Pathophysiology of thrombosis in MPN is complex and involves abnormalities in blood cells, plasma factors, and endothelial cells (ECs). Several groups, using different techniques, have shown *JAK2V617F* expression in endothelial cells (Supplemental Fig. 1, <http://links.lww.com/HS/A79>). Using laser capture microdissection, *JAK2V617F* was demonstrated in ECs from hepatic venules in 2 of 3 patients with PV and BCS.² *JAK2V617F* endothelial cells were demonstrated in microdissected splenic capillaries and in ECs cultured from

splenic vein in patients with myelofibrosis but without SVT.³ Although these teams performed experiments to ensure that the DNA they obtained originated from ECs, it is difficult to completely rule out a possible contamination by blood cells. Analysis of endothelial progenitor cells, specifically endothelial colony forming cells (ECFCs), is an alternative way to look for *JAK2V617F* ECs. Indeed, ECFCs are reported to be the only “true” endothelial progenitor cells, as they are the only ones able to generate blood vessels in vivo: they display clonogenic potential, endothelial but not myeloid cell surface markers, and pronounced postnatal vascularisation ability in vivo.^{4,5} ECFCs are a unique tool to investigate endothelial molecular dysfunction in disease, as they give access to endothelial cells from patients in a non-invasive way and a promising tool for vascular regenerative approaches and gene therapy.⁶ Yoder et al studied 11 *JAK2V617F* MPN patients and reported 3 *JAK2V617F* ECFCs derived from only 1 of 11 patients. Of note, this patient presented with thrombosis and later developed PV.⁴ In another study, the *JAK2V617F* mutation was not detected in any of 75 ECFCs obtained from 57 patients with *JAK2V617F* MPN but no thrombosis.⁷ Teofili et al reported *JAK2V617F* ECFCs in 5 of 22 MPN patients, all with thrombotic complications including 1 with BCS and 1 with PVT.⁸ Lastly, 4 of 5 *JAK2V617F*-positive patients with BCS but without overt MPN had *JAK2V617F* ECFCs cultured from the bone marrow.⁹ Taken together, these results suggest that the presence of *JAK2V617F* ECFCs in patients is associated with thrombosis, even in the absence of overt MPN. Our groups have previously demonstrated (a) that the presence of *JAK2V617F* in ECs modifies their phenotype and makes them prothrombotic,¹⁰ highlighting the importance of looking for *JAK2V617F* ECs in patients; (b) the importance of using correctly characterized ECFCs in investigating this.⁶ Confirming that *JAK2V617F* positive ECFCs are associated with previous thrombosis in MPN patients would suggest that ECFCs culture and *JAK2V617F* genotyping may be used as a marker of thrombotic risk in MPN patients, before they develop thrombosis.

The aim of our study was to analyze ECFCs isolated from peripheral blood of patients with Ph-negative *JAK2V617F*-positive MPN and to compare the results to the conclusions of previous studies. We focused on patients with *JAK2V617F* MPN and SVT, as these thrombotic complications are known to be

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closely associated with MPN. We describe results from 31 patients from Bordeaux, France and then London, United Kingdom (cohort 1: 20 patients) and London (cohort 2: 11 patients). (Table 1). All patients gave informed consent.

Cohort 1: ECFCs were cultured from 25 to 60 ml of peripheral blood (PB) as previously published.^{4,11-13} ECFCs appeared between 5 and 20 days of culture in 9/20 patients. In 7/9 patients, there was amplification to passage 4 (P4). ECFCs were characterized using morphologic criteria (monolayers of cobblestone-appearing cells) and their capacity to proliferate and to amplify until P4. Cloning cylinders were used to isolate each colony. After amplification of each colony separately, DNA and in 3 samples RNA were extracted. Reverse transcription quantitative polymerase chain reaction experiments (RT-qPCR) confirmed that the isolated ECFCs expressed endothelial markers (VWF, KDR, CD31, CD146) but not hematopoietic factors (CD45, CD14) (Supplemental Fig. 2A, <http://links.lww.com/HS/A80>). The presence of *JAK2V617F* was then investigated using quantitative allele-specific PCR techniques.^{14,15} Given that we analyzed each colony separately in cohort 1, we considered that a *JAK2V617F* positive colony would have an allele burden of 50% if heterozygous or 100% if homozygous.

Cohort 2: PB samples (60 ml) were collected from 11 patients for ECFCs isolation.^{4,11-13} ECFCs appeared between 8 and 28 days of culture in 9/11 patients. In 5/9 patients there was successful colony expansion until P4. ECFC were characterized in 3 patients using immunofluorescence for endothelial markers (VE-cadherin and vWF)¹³ and clonogenic capability for at least P4 (Supplemental Fig. 2B, <http://links.lww.com/HS/A80>).

Using identical methods in the 2 cohorts, we successfully isolated ECFCs in 18/31 patients. In 12 patients (38.7%) we could grow highly proliferative ECFCs colonies and expanded them for at least 4 passages. EC were characterized by immunofluorescence or RT-PCR in 5 patients. We obtained DNA of adequate quality from these cells (Fig. 1). In all 12 patients, the ECFCs carried the *JAK2* wild-type allele but not the mutated one (Table 1).

In summary, we studied 31 patients with MPN-SVT for *JAK2V617F* in ECFCs. ECFCs were grown using rigorous laboratory methods. Of the 12 patients where DNA from ECFCs was extracted and studied, none demonstrated the presence of *JAK2V617F* mutation in endothelial cells. This is in contradiction with previously published results.^{8,9} These differences may be explained by several reasons.

- (1) Culture conditions and definition of ECFCs could be different between our study and the others. Helman et al used BM as a source of endothelial cells and a methodology different of the standard procedure. Interestingly, they observed the appearance of colonies only after 7 days in culture, earlier than what observed from peripheral blood ECFCs. Thus, their method may have resulted in the isolation of an earlier or different type of endothelial progenitor cell, compared to circulating ECFCs. However, the culture conditions and definitions in our study are similar to those used in Teofili et al, and Yoder et al.
- (2) The *JAK2V617F* genotyping technique and threshold for *JAK2V617F* positivity can also have influenced the interpretation of the data. Indeed, in cohort 1, we considered that an ECFC carried *JAK2V617F* if the allele burden was 50% or 100%, as described above, given that the cells were isolated from single colonies. This same reasoning had been used by the Yoder group who reported that the 3 *JAK2V617F* ECFC

they found in the same patient carried the mutation at the heterozygous state.⁴ In cohort 2, where we did not isolate single clones, we used a threshold of 1%. It may be that other reports used a lower *JAK2V617F* positive threshold, which can lead to the risk of detecting “contaminating” *JAK2V617F* positive blood cells that do not belong to the ECFCs clone.

- (3) The differences may also be due to patients’ characteristics. Indeed, although all our patients had overt MPN and SVT, the analysis of ECFCs occurred at various time points after diagnosis of MPN and initiation of cytoreductive treatment. Notably our cohort comprised a range of MPN diagnoses and cytoreductive agent. However, a third of our patients were assessed before initiation of cytoreductive therapy, none of them presenting *JAK2V617F* positive ECFCs.
- (4) We could only grow highly proliferative ECFCs (up to passage 4) for 12/31 patients (38.7%), all being *JAK2* wild type. Recently, the Vascular Biology Standardization Subcommittee from the International Society on Thrombosis and Hemostasis published standardization of methods to quantify and culture ECFCs. They reported a success rate of 70% in isolating ECFC from peripheral blood of healthy subjects, keeping in mind that ECFC colonies were defined as well-circumscribed colonies of cobblestone appearance with more than 50 adherent cells. Here, our success rate is lower; but we only reported the growth of ECFC that had undergone at least 4 passages, as we aimed to have sufficient number of ECFCs to obtain DNA of good enough quality. Besides, we cannot completely rule out that, for unexplained reasons, we could not grow *JAK2V617F* ECFCs but only *JAK2* wild type ECFCs.
- (5) Among the 12 informative MPN-SVT patients, 10 had Portal Vein thrombosis and only 2 had Budd Chiari Syndrome. Whereas we can confidently conclude that *JAK2V617F* ECFCs are not a hallmark of PVT, it is hard to draw the same conclusion for BCS as we only analyzed 2 patients.

In conclusion, using well-established methods across two cohorts of MPN-SVT patients we did not find any circulating *JAK2V617F* ECFCs in these patients. The differences between this and other studies raise the question of whether the methodology for isolation and characterization of ECFCs may influence the findings. The presence of the *JAK2V617F* mutation in ECs could have a significant role in the thrombotic pathophysiology of the disease; hence ECFCs from peripheral blood might be used as a marker of thrombotic risk in MPN patients. Our results suggest that this is not always the case and that this technique is currently not sufficiently reproducible to define patients with thrombotic risk. Our results do not exclude the presence of endothelial-like cells derived from the hematopoietic lineage, expressing *JAK2V617F*, which can integrate into the vessel wall. This would explain the presence of *JAK2V617F* endothelial cells found using microdissection in the spleen and liver.

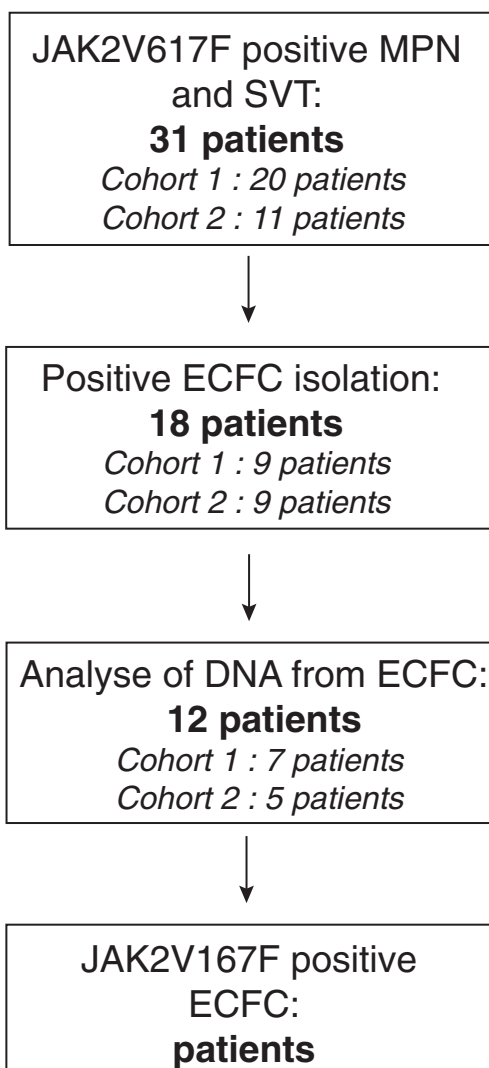
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Table 1**Characteristics of the 12 Patients With SVT and ECFCs Analyzed.**

Patient	Age	Gender	MPN	Site of thrombosis	Blood count at diagnosis of thrombosis			Anomalies at thrombophilia testing	Other site of thrombosis	Time between thrombosis and ECFCs analysis (months)	Cytoreductive therapy at the time of ECFCs analysis	Number of		ECFCs status	
					Allelic burden	Haematocrit (%)	Leukocytes (G/L)					Platelets (G/L)	Number of ECFCs		ECFCs analysed*
<i>Cohort 1</i>															
1	59	M	ET	Portal vein thrombosis	7	45.4	5.2	208	None	Deep venous thrombosis	1	No	3	3	WT
2	51	M	ET	Portal vein thrombosis	45	42.2	5.5	347	NA	None	1	HU	1	1	WT
3	31	F	ET	Portal vein thrombosis	13	NA	NA	NA	NA	Splenic vein thrombosis	36	IFN	1	1	WT
4	44	F	PV	Portal vein thrombosis	51	NA	NA	NA	NA	Splenic infarct	144	HU	1	1	WT
5	41	F	PV	Portal vein thrombosis	28	32.5	5.3	352	NA	None	80	Ruxolitinib	5	4	WT
6	65	F	PV	Portal vein thrombosis + splenic vein thrombosis	56	38.1	6.5	184	IgM anti-cardiolipin	None	6	No	1	1	WT
7	50	F	PMF	Budd-Chiari syndrome	8	41.3	7.4	448	None	None	2	No	1	1	WT
<i>Cohort 2</i>															
8	50	F	MPN-U	Portal vein thrombosis	N/A	40.1	5.49	258	Prothrombin gene mutation heterozygous	Splenic vein and superior mesenteric vein thrombosis	60	No	1	N/A	WT
9	35	F	ET	Portal vein thrombosis	N/A	43.7	11.32	495	MTHFR heterozygous	Splenic vein thrombosis	48	HU	24	N/A	WT
10	39	F	ET	Portal vein thrombosis	N/A	43.8	11	593	None	Splenic vein thrombosis	108	Ruxolitinib	1	N/A	WT
11	50	M	ET	Budd-Chiari syndrome	N/A	40.7	14.5	692	Elevated FVIII	None	6	HU	3	N/A	WT
12	53	F	PV	Portal vein thrombosis	N/A	30.4	5.17	412	None	Splenic vein thrombosis	84	Ruxolitinib	2	N/A	WT

ECFCs = endothelial colony-forming cells, ET = essential thrombocythemia, HU = Hydroxyurea, IFN = Interferon, MF = primary myelofibrosis, MPN = myeloproliferative neoplasm, NA = non available, PV = polycythemia vera, WT = wild-type.

**Figure 1. Flow-chart of patients.**

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Supplemental methods, <http://links.lww.com/HS/A81>

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