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

Alexandre Guy1,2, Anicee Danaée3, Koralia Paschalaki4, Lisa Boureau2, Etienne Rivière1,5, Gabriel Etienne6, Olivier Mansier1,2, Michael Laffan4, Mallika Sekhar3, Chloe James1,2

Correspondence: Chloe James (e-mail: chloe.james@inserm.fr); Mallika Sekhar (e-mail: mallika.sekhar@nhs.net).

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/HSS/A79). Using laser capture microdissection, JAK2V617F was demonstrated in ECs from hepatic venules in 2 of 3 patients with JAK2V617F MPN but no JAK2V617F- positive ECFCs were demonstrated in microdissected splenic capillaries and in ECs cultured from splenic vein in patients with myelofibrosis but without SVT.2 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 5 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 PV and 1 with BCS.8 Lastly, of 4 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.10 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
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.413-13 ECFCs appeared
between 5 and 20 days of culture in 9/20 patients. In 79 patients,
there was amplification to passage 4 (P4). ECFCs were
characterized using morphologic criteria (monolayers of cobble-
stone-appearing cells) and their capacity to proliferate and to
amplify until P4. Cloning cylinders were used to isolate each
colonies. 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 ECFC isolation.511-13 ECFCs appeared between 8 and 28
days of culture in 9/11 patients. In 8/9 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 ECFC 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 interpre-
tation 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
may 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 Sub-
committee 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 30 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
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.

Acknowledgements

Bordeaux: We wish to thank Axelle Lacaux, Jean-François
Viallard, Pierre Duffau for their help in patient recruitment. We
are also grateful to the French Intergroup Myeloproliferative
(FIM). This study was supported by research grants from
INSERM, ANR-DFG JAKPOT and The Fondation Bettencourt
Schueller. AG was supported by research grant from INSERM
(Poste Accueil INSERM).
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 | Other site of thrombosis | Time between thrombosis and ECFC analysis (months) | Cytoreductive therapy at the time of ECFCs analysis | Number of ECFCs analyzed | Number of wild-type | Allelic mutation status |
|---------|-----|--------|-----|-------------------|----------------------------------------|---------------------------|-----------------------------------------------|-----------------------------------------------|------------------------|---------------------|-----------------------|
| 1       | 59  | M      | ET  | Portal vein thrombosis | 45.4 | 5.2 | 308 | None | Deep venous thrombosis | 1 | No | 3 | WT |
| 2       | 51  | M      | ET  | Portal vein thrombosis | 46  | 5.5 | 347 | NA | None | 1 | HU | 1 | WT |
| 3       | 31  | F      | ET  | Portal vein thrombosis | 13  | NA | NA | NA | Splenic vein thrombosis | 36 | IFN | 1 | WT |
| 4       | 44  | F      | PV  | Portal vein thrombosis | 51  | NA | NA | NA | Splenic vein thrombosis | 144 | HU | 1 | WT |
| 5       | 41  | F      | PV  | Portal vein thrombosis | 28  | 3.25 | 352 | NA | None | 80 | Ruxolitinib | 5 | WT |
| 6       | 65  | F      | PV  | Portal vein thrombosis | 58  | 3.81 | 184 | IFN | anti-cardiolipin | 6 | No | 1 | WT |
| 7       | 50  | F      | PMF | Budd-Chiari syndrome | 41.3 | 7.4 | 446 | None | None | 2 | No | 1 | WT |
| 8       | 35  | F      | PV  | Portal vein thrombosis | 43.7 | 5.3 | 593 | MTHFR heterozygous | Splenic vein thrombosis | 158 | Ruxolitinib | 2 | WT |
| 9       | 39  | F      | ET  | Portal vein thrombosis | 43.6 | 5.49 | 318 | None | None | 6 | No | 1 | WT |
| 10      | 30  | M      | ET  | Budd-Chiari syndrome | 40.7 | 14.5 | 692 | Blunted FVIII | None | 6 | No | 1 | WT |
| 11      | 50  | F      | PV  | Portal vein thrombosis | 30.4 | 5.17 | 412 | None | Splenic vein thrombosis | 84 | Ruxolitinib | 2 | 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.

References
1. Sekhar M, McVinnie K, Burroughs AK. Splanchnic vein thrombosis in myeloproliferative neoplasms. Br J Haematol. 2013;162:730–747.
2. Sozer S, Fiel MI, Schiano T, et al. The presence of JAK2V617F mutation in the liver endothelial cells of patients with Budd-Chiari syndrome. Blood. 2009;113:5246–5249.
3. Rosti V, Villani L, Riboni R, et al. Spleen endothelial cells from patients with myelofibrosis harbor the JAK2V617F mutation. Blood. 2013;121:360–368.
4. Yoder MC, Mead LE, Prater D, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood. 2007;109:1801–1809.
5. Medina RJ, Barber CL, Sabatier F, et al. Endothelial progenitors: a consensus statement on nomenclature: endothelial progenitor nomenclature. Stem Cells Transl Med. 2017;6:1316–1320.
6. Paschalaki KE, Randi AM. Recent advances in endothelial colony forming cells toward their use in clinical translation. Front Med. 2018;5:295.
7. Piaggio G, Rosti V, Corselli M, et al. Endothelial colony-forming cells from patients with chronic myeloproliferative disorders lack the disease-specific molecular clonality marker. Blood. 2009;114:3127–3130.
8. Teofili L, Martini M, Iachininoto MG, et al. Endothelial progenitor cells are clonal and exhibit the JAK2V617F mutation in a subset of thrombotic patients with Ph-negative myeloproliferative neoplasms. Blood. 2011;117:2700–2707.
9. Helman R, Pereira W, de O, et al. Granulocyte whole exome sequencing and endothelial JAK2V617F in patients with JAK2V617F positive Budd-Chiari Syndrome without myeloproliferative neoplasm. Br J Haematol. 2018;180:443–445.
10. Guy A, Gourdou-Latyszenko V, Lay NL, et al. Vascular endothelial cell expression of JAK2V617F is sufficient to promote a pro-thrombotic...
state due to increased P-selectin expression. Haematologica. 2019;104:70–81.
11. Ingram DA, Mead L, Tanaka H, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood. 2004;104:2752–2760.
12. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. Blood. 2011;117:1071–1080.
13. Paschalaki KE, Starke RD, HU Y, et al. Dysfunction of endothelial progenitor cells from smokers and chronic obstructive pulmonary disease patients due to increased DNA damage and senescence. Stem Cells. 2013;31:2813–2826.
14. Larsen TS, Christensen JH, Hasselbalch HC, et al. The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders. Br J Haematol. 2007;136:745–751.
15. Lippert E, Boissinot M, Kralovics R, et al. The JAK2-V617F mutation is frequently present at diagnosis in patients with essential thrombocythemia and polycythemia vera. Blood. 2006;108:1865–1867.