ARTÍCULO 3

"Monitorización de células plasmáticas tumorales circulantes en sangre periférica de pacientes con mieloma múltiple tras tratamiento mediante citometría de flujo de nueva generación"

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Blood, (2019), 134:24
DOI: 10.1182/blood.2019002610.

La información suplementaria correspondiente a este trabajo y citada en el mismo está disponible en formato electrónico en la página web de la revista, así como en el Anexo III y en el disco compacto disponibles al final de esta memoria de tesis doctoral.

Introducción. La técnica de citometría de flujo de nueva generación se ha desarrollado recientemente como herramienta de elevada sensibilidad e importante valor pronóstico, tanto para la detección de células plasmáticas tumorales circulantes (CPTC) en sangre al diagnóstico en pacientes con neoplasias de CP, como para la monitorización de enfermedad mínima residual (EMR) en médula ósea (MO) de pacientes con mieloma múltiple (MM) tras tratamiento. No obstante, hasta la fecha no se ha investigado la utilidad de la citometría de flujo de nueva generación para detectar la presencia de CPTC en sangre de pacientes con MM una vez administrado el tratamiento, y su posible utilidad pronóstica adicional respecto a la presencia de EMR en MO y/o la persistencia en suero del componente monoclonal.

Objetivo. Analizar la frecuencia de pacientes con MM que, tras tratamiento, presentaban CPTC en sangre mediante citometría flujo de nueva generación, y su posible asociación tanto con la persistencia de EMR en MO y de componente monoclonal (CM) en suero por inmunofijación, como con un posible impacto pronóstico.

Materiales y métodos. En este trabajo, aplicamos la técnica de citometría flujo de nueva generación sobre 274 muestras pareadas de sangre periférica y MO provenientes de 137 pacientes tratados con MM, analizando, además, en un subgrupo de 54 pacientes, muestras secuenciales de sangre periférica (>1 estudio). A nivel pronóstico, evaluamos el impacto de la presencia de CPTC en sangre sobre la supervivencia de estos pacientes, respecto a los factores de riesgo conocidos más relevantes como la edad, el perfil citogenético estudiado mediante hibridación *in situ* fluorescente (FISH) sobre núcleos interfásicos de poblaciones purificadas de CP, y la persistencia/ausencia de EMR en MO o de CM en suero por inmunofijación.

Resultados. En conjunto, 36 de los 137 (26%) pacientes con MM analizados tras tratamiento mostraron CPTC en sangre. Merece destacar que todos ellos presentaron, además, persistencia de EMR positiva en MO (36/36), detectándose en más de la mitad (21/36, 58%) la presencia de componente monoclonal en suero. Pese a estos resultados, la sensibilidad de la detección de CPTC en sangre a la hora de identificar enfermedad persistente en pacientes tratados con MM, fue inferior a la obtenida con los estudios de EMR en MO (66%; 91/137) y de inmunofijación en suero (45%; 62/137). Aun así, la presencia de CPTC en sangre constituyó un factor pronóstico adverso e independiente de otros parámetros, a la hora de predecir la de supervivencia libre de progresión de los pacientes estudiados, tanto en su conjunto -índice de riesgo de 5,1 intervalo de confianza (IC) del 95%: 2,9-8,9; p<0,0001-, como de forma específica en aquellos casos que

alcanzaron respuesta completa (RC) o RC estricta -índice de riesgo de 7,4 (IC del 95%: 3,0-18,2; p<0,0001)-. Además, cabe señalar que el valor predictivo de la presencia de CPTC en sangre no solo era independiente de la respuesta clínica alcanzada, sino también del momento del tratamiento en el que se evaluó su presencia (durante el tratamiento *vs.* final de tratamiento). Finalmente, la ausencia de CPTC en sangre en dos o más estudios consecutivos demostró ser la variable que mejor refleja el comportamiento evolutivo de la enfermedad, facilitando la optimización del seguimiento clínico a largo plazo de los pacientes con MM, al permitir discriminar de forma más clara entre pacientes con bajo *vs.* alto riesgo de recaída.

Conclusiones. La presencia de CPTC detectada mediante citometría flujo de nueva generación en sangre de pacientes con MM tras tratamiento, constituye un marcador de elevado valor pronóstico que refleja de forma constante la persistencia de EMR en MO. Además, permite una monitorización estrecha de los pacientes con MM empleando procedimientos mínimamente invasivos, particularmente en aquellos casos que han finalizado la terapia activa. Desde el punto de vista pronóstico, la presencia de CPTC en sangre de pacientes con MM tras tratamiento proporciona información complementaria a la de otros factores pronósticos bien establecidos, a la hora de evaluar la respuesta al tratamiento, permitiendo identificar un subgrupo de pacientes con riesgo inminente de progresión, independientemente de la calidad de la respuesta clínica y de los niveles de EMR en MO alcanzados con el tratamiento.

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TO THE EDITOR:

Blood monitoring of circulating tumor plasma cells by next generation flow in multiple myeloma after therapy

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In recent years, detection of circulating tumor plasma cells (CTPC), tumor cell–derived deoxyribonucleic acid (DNA), RNA, or protein markers in blood has gained interest for disease monitoring in multiple myeloma (MM).[1,2] This is mainly because of (1) the minimally invasive nature of blood vs bone marrow (BM) analyses, (2) the possibility for more precise quantification of absolute numbers of CTPC than BM minimal residual disease (MRD) resulting from absence of potential hemodilution, and (3) the (nonlinear) correlation observed between CTPC numbers and BM disease burden at diagnosis.[1,3] Recently, we have shown by high-sensitivity next-generation flow (NGF) that CTPC are systematically present in blood of MM at diagnosis, with an adverse prognostic impact for higher counts.[3] These results highlight the relevance of greater levels of disease dissemination via blood in conferring a malignant behavior to MM, suggesting the presence of blood CTPC might be required for subsequent disease progression of treated MM patients.

Based on this hypothesis, here we investigate for the first time the prognostic impact of CTPC by NGF in blood of 137 newly diagnosed MM patients after active treatment outside clinical trials (supplemental Table 1 on the *Blood* Web site), in parallel to BM MRD and serum immunofixation (sIF). Overall, a total of 328 samples were analyzed: 274 paired BM and blood samples, plus 54 follow-up blood specimens. Following the EuroFlow-NGF MM MRD approach,[4] a median (range) of 6 mL (3-14 mL) of blood and 1.8 mL (0.3-5 mL) of BM sample were lysed to (systematically) obtain $\geq 10^7$ cells per sample. In parallel, sIF was measured by the HYDRAGEL kit (HYDRASYS system, Sebia, Barcelona, Spain).[5] Statistical significance was set at P values $< .05$ (supplemental Materials). All studies were approved by the institutional review board.

Following therapy, persistence of CTPC in blood was detected in 26% of MM cases. This represents a 50% higher frequency than previously reported by conventional flow cytometry (18%-19%),[6-8] reaching rates similar to those found with other high-sensitivity techniques such as allele-specific oligonucleotide polymerase chain reaction (25%-28.8%[9,10]) or next-generation sequencing (31%-34%[2,11] for cell-free DNA and 40%[2] for genomic leukocyte DNA). This translated into even higher differences among patients who reached complete response (CR)/stringent CR (sCR): 17% CTPC$^+$ cases in our series vs 0%[12,13] to <8%[6,8] in other previous conventional flow cytometry studies (supplemental Table 2).

Despite the greater sensitivity and rate of positivity for CTPC reported here, a significant proportion of our MM cases that were BM MRD$^+$ or sIF$^+$ still had undetectable CTPC in (paired) blood samples: 55/137 (40%) and 41/137 (30%), respectively. In contrast, 15/36 (42%) CTPC$^+$ cases were also sIF$^-$ (supplemental Table 2). These findings indicate that CTPC is a less sensitive MRD marker in MM than BM MRD, complementary to sIF, in line with previous observations.[1] However, although BM MRD and sIF mainly reflect persistence of resistant tumor[14] and tumor cell–derived immunoglobulins,[15] they fail to provide insight on the ability of these cells to support tumor regrowth and/or dissemination, which ultimately determine disease progression. In contrast, CTPC might not only reflect tumor load but, particularly, the ability of persisting tumor cells to disseminate the disease and support tumor growth and progression at (multiple) distant sites in BM and other tissues, as previously suggested[16] based on their more immature and prominent stem cell-like PC features compared with (paired) BM-derived tumor-plasma cells (TPC).[3]

Despite all of this, every CTPC$^+$ case in our cohort was BM MRD$^+$, suggesting that the presence of blood CTPC after therapy might be a surrogate marker of persistent BM MRD in guiding (eg, avoiding) subsequent (more invasive) BM aspiration

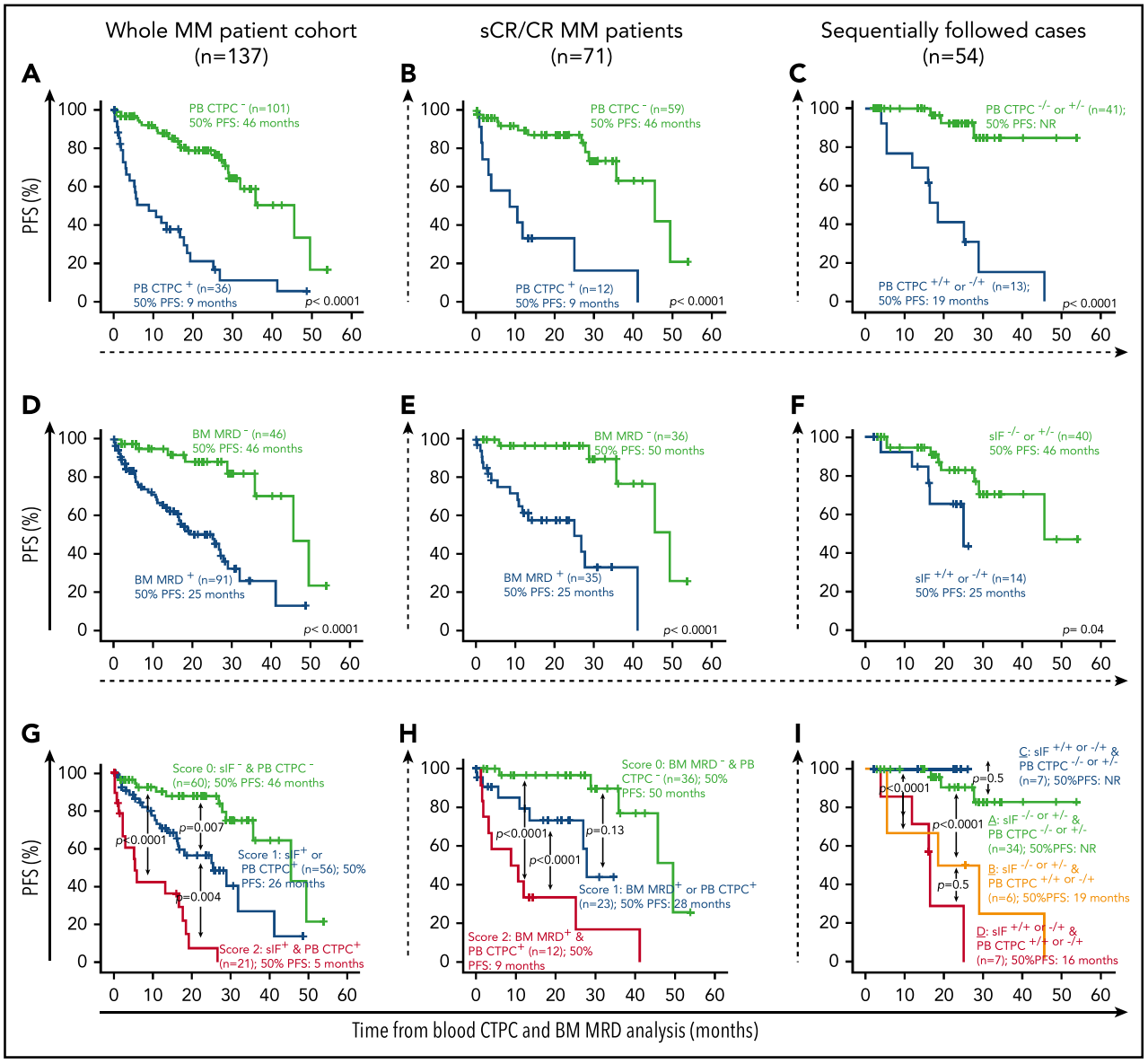

Figure 1. Prognostic impact of blood CTPC by NGF (vs BM MRD and sIF) on PFS of MM patients according to patient response to therapy. (A-B) Effect of PB CTPC, (D-E) BM MRD, (H) combination of both parameters, and (G) PB CTPC together with sIF status on PFS is displayed for (A, D, G) the entire MM cohort and (B, E, H) for sCR and CR patients, respectively. PFS curves of MM patients grouped according to (C) their sequential PB CTPC ($^{-/-}$ or $^{+/-}$ vs $^{-/+}$ and $^{+/+}$), (F) sIF status, or (I) a combination of both, are shown. Overall, CTPC^{-} and MRD^{-} was defined as the absence of TPC in PB or BM by NGF, respectively, with a limit of detection of $<2 \times 10^{-6}$. CR, complete response; NR, not reached; PB, peripheral blood.

procedures, particularly among sCR/CR patients. In contrast, a significant fraction of our CTPC^{-} cases were BM MRD^{+} and/or sIF^{+}, supporting the notion that MM is a BM disease with greater levels of infiltration by (usually) functional PC in BM vs PB. Prolonged half-life (~23 days) and complete clearance (~29 weeks) of the M-protein for the most prevalent immunoglobulin G subclass,[17] in addition to persistence of extramedullary disease[18] and/or the administration of monoclonal antibody-therapy (eg, daratumumab)[19] for MM patients, might also explain sIF positivity in at least a subset of BM MRD^{-}/sIF^{+} cases. Additionally, poor BM sample quality (eg, from hemodilution) might also play a role because abnormally low (≤0.002%)[4] mast cell counts were detected here in 5/10 BM MRD^{-}/sIF^{+} cases. In contrast, sIF negativity among 4 of our non-sCR/CR patients could be related to the appearance/persistence of plasmacytomas[18] (2/4 cases), and high free light chain ratio levels (>500) without measurable M-component in serum and urine[18] (1/4 cases), together with a non-secretory TPC[15] detectable here in another MM patient.

From the prognostic point of view, our results based on real-world MM show for the first time that the absence vs presence of blood CTPC by NGF is a new powerful independent prognostic marker for progression-free survival (PFS) measured from the time of BM-MRD/CTPC assessment both among the entire MM patient cohort (hazard ratio [HR], 5.1; 95% confidence interval [CI], 2.9-8.9; $P < .0001$) (Figure 1A) and within sCR/CR cases (HR, 7.4; 95% CI, 3.0-18.2; $P < .0001$; Figure 1B), complementary to currently available prognostic tools such as sIF and BM MRD, respectively (Table 1), and regardless of the treatment phase BM-MRD/CTPC being assessed (supplemental Figure 1).

Table 1. Multivariate analysis of prognostic factors For PFS In MM

	Univariate analysis		Multivariate analysis		
	Median PFS (mo)	*P*	HR	(95% CI)	*P*
Prognostic factors for entire MM series					
Age					
<65 y	28	.3	—	—	—
≥65 y	36				
Cytogenetic profile by FISH					
Standard-risk	36	.07	—	—	—
High-risk	16				
Serum IF					
Negative	41	.001	—	—	—
Positive	18		2.4	(1.3-4.4)	.004
BM MRD status by NGF					
Negative	46	<.0001	—	—	—
Positive	25				
PB CTPC status by NGF					
Negative	46	<.0001	—	—	—
Positive	9		5.1	(2.9-8.9)	<.0001
Prognostic factors for sCR/CR cases					
Age					
<65 y	50	.5	—	—	—
≥65 y	41				
Cytogenetic profile by FISH					
Standard-risk	50	.09	—	—	—
High-risk	28				
BM MRD status by NGF					
Negative	50	<.0001	—	—	—
Positive	25		6.1	(1.5-24.4)	.01
PB CTPC status by NGF					
Negative	46	<.0001	—	—	—
Positive	9		7.4	(3.0-18.2)	<.0001

High-risk cytogenetics was defined as presence at diagnosis of t(4;14); t(11;14); t(14;16); 1q amplification, deletion 13q, and/or deletion 17p. Standard-risk cytogenetics includes all other cases. (Data available in 96/137 cases.)

FISH, fluorescent in situ hybridization; IF, immunofixation.

Based on those covariables that showed a (statistically) significant effect on PFS in multivariate analysis (Table 1), a prognostic score was built that allowed identification of a subgroup of blood CTPC^{+} MM patients with a very poor outcome (score 2 in the risk stratification models proposed here) with PFS rates at 2 years of only 1% for the entire patient cohort (Figure 1G) and of 33% for sCR/CR MM patients (Figure 1H), respectively.

These results, together with the demonstration that CTPC are systematically detected in blood of MM at diagnosis[3] and at relapse,[13] suggest that detection of blood CTPC rather than a surrogate marker of response is a strongly reliable predictor of impending (early) disease progression[9] (Figure 1A-B). In contrast, BM MRD would be a better predictor of persistent disease (Figures 1D-E) and longer term prognosis of MM undergoing different therapies, as recurrently shown in the literature in the settings of clinical trials, both for conventional[20-22] and for high-sensitivity NGF[4,23] and next-generation sequencing[24] approaches. However, frequent BM sampling is hampered by the invasive nature of BM aspiration procedures,[1] whereas more frequent follow-up of CTPC in blood is feasible. Thus, sequential monitoring of blood CTPC was performed in a subset of 54 cases in parallel to sIF. Our results showed that MM patients who were persistently CTPC^{-} (CTPC$^{-/-}$) or that became CTPC^{-} after being CTPC^{+} (CTPC$^{+/-}$) showed a significantly better outcome than cases with a blood CTPC^{+} result in the last follow-up study (CTPC$^{+/+}$ or $^{-/+}$) (Figure 1C), independent of sIF status (Figure 1F, I). However, this should be confirmed in larger series of patients with longer monitoring because the limited number of sIF$^{+/+}$ or $^{-/+}$ (n = 7) cases was observed for a paradoxically (slightly) better outcome vs the sIF$^{-/-}$ or $^{+/-}$ (n = 34) MM showing no CTPC in the last follow-up study (ie, CTPC$^{-/-}$ or $^{+/-}$ cases) (Figure 1I). In spite of that, the former findings suggest that blood CTCP might provide additional relevant prognostic information to single time-point BM MRD assessment in predicting for longer term outcome in patients that either (persistently) remain or turn CTPC^{-} in sequential follow-up studies, in line with previous reports.[9,25]

In summary, we show here that blood CTPC is a novel independent prognostic marker for PFS in real-world MM prone to more frequent monitoring, which provides early indication of impending disease progression, regardless of BM MRD and sIF status. These results suggest that presence of blood CTPC

of MM after therapy probably reflects a unique distinct tumor biology (eg, tumor dissemination capacity) at a given time point after therapy with important clinical consequences. Further studies in larger series of MM patients outside and inside clinical trials are required to confirm our findings.

Acknowledgments

The authors thank Sergio Matarraz and Lourdes Martín-Martín for their support on statistical analysis.

This work has been supported by the International Myeloma Foundation-Black Swan Research Initiative and the EuroFlow Consortium; Centro de Investigación Biomédica en Red de Cáncer (CIBER-ONC; Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Madrid, Spain, and FONDOS Europeo de Desarrollo Regional) grants CB16/12/00400 (L.S.-F., J.F.-M., A.C.-M., and A.O.), CB16/12/00233 (N.P., O.G.-S., M.D.-C., M.G. and M.-V.M.), CB16/12/00369 and CB16/12/00489 (B.P. and J.S.M.); grant SA079U14 from the Consejería de Educación, Junta de Castilla y León, Valladolid, Spain; and grant DTS15/00119 from the Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Madrid, Spain; by the Research Foundation of the State of Rio de Janeiro, Rio de Janeiro, Brazil (grant E26/110.105/2014) and Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPQ of Brazil (grants 303765/2018-6 and 409440/2016-7).

The EuroFlow Consortium is an independent scientific consortium that aims at innovation and standardization of diagnostic flow cytometry. All consortium members that contributed are listed as authors. All acquired knowledge and experience within EuroFlow is shared with the scientific and diagnostic community after protection of the relevant Intellectual Property; for example, by filling patents. The involved patents are owned by the EuroFlow Consortium and licensed to companies, including Cytognos SL (Salamanca, Spain), Becton/Dickinson Biosciences (San José, CA), and Immunostep SL (Salamanca, Spain). The revenues of the patents are exclusively used for EuroFlow Consortium activities, such as for covering (in part) the costs of the Consortium meetings, the EuroFlow Educational Workshops, and the purchase of custom-made reagents for collective experiments. J.F.-M., J.J.M.v.D., and A.O. are part of the inventors on the EuroFlow-owned patent PCT/NL/2013/050420; US 62/072 498 (Methods, reagents and kits for detecting minimal residual disease). This patent is licensed to Cytognos, which pays royalties to the EuroFlow Consortium.

Authorship

Contribution: L.S.-F., T.C.S., R.P., A.C.-M., and O.G.S. performed experiments; L.S.-F. and J.F.M. analyzed results; N.P., M.D.-C., R.J.P.d.M., L.G.-M., J.M.A.-A., A.G.-M., C.A.-F., J.L., A.B.-G., A.M., E.S.d.C. and M.G. contributed to the data collection and performed patients management; L.S.-F., J.F.-M., B.P., J.S.M., M.V.M., B.D., J.J.M.v.D., and A.O. designed the research; and L.S.-F., J.F.-M. and A.O. wrote the paper.

Conflict-of-interest disclosure: M.-V.M. declares honoraria for lectures and advisory boards from Janssen, Celgene, Amgen, Takeda, Abbvie, GSK, Adaptive, EDO-Mundipharma, and Pharmamar. The remaining authors declare no competing financial interests.

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Footnotes

For original data, please contact orfao@usal.es.

The online version of this article contains a data supplement.

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DOI 10.1182/blood.2019002610

TO THE EDITOR:

Clinical utility of targeted next-generation sequencing–based screening of peripheral blood in the evaluation of cytopenias

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One of the most common reasons for hematology consultation is the evaluation of cytopenia(s). The workup of patients who present with cytopenia(s) can be extensive, given the wide differential diagnosis.[1] Although only a minority of patients are ultimately diagnosed with a hematologic malignancy, a key entity to exclude in this differential is myelodysplastic syndrome (MDS),[2] which requires bone marrow morphology and cytogenetics for diagnosis. There is a clinical need for the development of minimally invasive ancillary tests to enhance conventional hematologic workup (eg, complete blood count with differential, B12/folate testing, iron-related studies, and serum protein electrophoresis) in the identification of patients who are at a low risk of having an underlying hematologic malignancy as the cause of cytopenia(s), thereby avoiding a costly and invasive bone marrow biopsy (BMBx).

Mutation profiling of peripheral blood (PB) using next-generation sequencing (NGS) is an attractive solution to this problem because of its potential application as a minimally invasive screen. Recent large-scale genome-sequencing studies using bone marrow samples have demonstrated that most cases of MDS and other related neoplasms, such as acute myeloid leukemia and myelodysplastic/myeloproliferative neoplasm overlap syndromes, harbor pathogenic somatic mutations in diverse myeloid cancer driver genes.[2-6] Moreover, some or all of these mutations can be detected in PB granulocytes in most patients with MDS and related neoplasms (Phillip D. Michaels, Dahai Wang, A.S.K., manuscript in preparation).[7,8]

Despite these advances and the widespread use of NGS testing, there are limited data on the clinical use of NGS testing in the early evaluation of patients with cytopenia(s). We hypothesize that targeted PB NGS using a myeloid cancer gene panel can be a valuable minimally invasive, ancillary tool in identifying patients with an underlying myeloid neoplasm as the cause of cytopenia(s). Herein, we report the clinical utility of PB screening by targeted NGS testing in a large institutional cohort of patients with cytopenia(s).

After institutional review board approval, we retrospectively identified all patients presenting with PB cytopenia(s) over a 30-month period (January 2015 through June 2017) to the Hematology Clinic at the Dana-Farber/Brigham and Women's